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Sir:

Transmitted herewith for filing is a continuation-in-part application filed under 37 CFR 1.53(b)(1) and claims priority to Serial No. NA filed April 7, 1997 entitled A SPHINGOMONAS BIODESULFURIZATION CATALYST by Aldis Darzins and Gregory T. Mrachko, the contents of which are incorporated herein by reference in its entirety.

Inventors: Aldis Darzins and Gregory T. Mrachko


Title: A SPHINGOMONAS BIODESULFURIZATION CATALYST☒ Specification, Claims, Abstract of the Disclosure☒ 24 sheets of ~~XXXXX~~/informal drawings. (Figs. 1A-1D, 2A-2C, 3A-3C, 4, 5, 6A-6G, 7, 8, 9, 10 and 11)☐ An assignment of the invention to _____☐ A verified statement to establish small entity status under 37 C.F.R. 1.9 and 37 C.F.R. 1.27.☒ ~~XXXXXXXX~~/Unexecuted Combined Declaration/Power of Attorney.☐ Other: _____

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Respectfully submitted,
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Dated: 5/5/97

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<p>Date: <u>May 5, 1997</u> EXPRESS MAIL LABEL NO. <u>EM080924599US</u></p>

Inventors: Aldis Darzins and Gregory T. Mrachko
Attorney's Docket No.: EBC97-06A

A *SPHINGOMONAS* BIODESULFURIZATION CATALYST

RELATED APPLICATIONS

This is a continuation-in-part application filed under
37 CFR 1.53(b) (1) and claims priority to Serial No. _____
5 filed April 7, 1997, entitled A *SPHINGOMONAS*
BIODESULFURIZATION CATALYST by Aldis Darzins and Gregory T.
Mrachko, the contents of which are incorporated herein by
reference in its entirety.

BACKGROUND OF THE INVENTION

10 The microbial desulfurization of fossil fuels has been
an area of active investigation for over fifty years. The
object of these investigations has been to develop
biotechnology based methods for the pre-combustion removal
of sulfur from fossil fuels, such as coal, crude oil and
15 petroleum distillates. The driving forces for the devel-
opment of desulfurization methods are the increasing levels
of sulfur in fossil fuel and the increasingly stringent
regulation of sulfur emissions. Monticello et al.,
"Practical Considerations in Biodesulfurization of

Petroleum," IGT's 3d Intl. Symp. on Gas, Oil, Coal and Env. Biotech., (Dec. 3-5, 1990) New Orleans, LA.

Many biocatalysts and processes have been developed to desulfurize fossil fuels, including those described in U.S. Patent Nos. 5,356,801, 5,358,870, 5,358,813, 5,198,341, 5,132,219, 5,344,778, 5,104,801 and 5,002,888, incorporated herein by reference. Economic analyses indicate that one limitation in the commercialization of the technology is improving the reaction rates and specific activities of the biocatalysts, such as the bacteria and enzymes that are involved in the desulfurization reactions. The reaction rates and specific activities (sulfur removed/hour/gram of biocatalyst) that have been reported in the literature are much lower than those necessary for optimal commercial technology. Therefore, improvements in the longevity and specific activity of the biocatalyst are desirable.

SUMMARY OF THE INVENTION

The invention relates to a novel microorganism, designated *Sphingomonas* sp. strain AD109, as well as isolated proteins and nucleic acid sequences obtained from this microorganism. This microorganism was obtained using a soil enrichment process using 2-(2-hydroxyphenyl)benzenesulfinate as the sole sulfur source. A biologically pure sample of this microorganism has been isolated and characterized.

The invention also relates to a collection of desulfurization enzymes isolated from *Sphingomonas* sp. strain AD109 which, together, catalyze the oxidative desulfurization of dibenzothiophene (DBT).

In another embodiment, the invention includes an isolated nucleic acid molecule, such as a DNA or RNA nucleotide sequence or molecule, which encodes one or more of the *Sphingomonas* desulfurization enzymes, or a homologue

or active fragment thereof. The invention also includes a recombinant microorganism containing one or more heterologous nucleic acid molecules which encode one or more of the *Sphingomonas* desulfurization enzymes or

5 homologues or active fragments thereof.

In a further embodiment, the invention provides a method of using the *Sphingomonas* microorganism or an enzyme preparation derived therefrom as a biocatalyst in the biocatalytic desulfurization of a fossil fuel containing
10 organosulfur compounds. The method comprises the steps of (1) contacting the fossil fuel with an aqueous phase containing a *Sphingomonas* biocatalyst which is capable of biocatalytic desulfurization and, optionally, a flavoprotein, thereby forming a fossil fuel and aqueous
15 phase mixture; (2) maintaining the mixture under conditions sufficient for sulfur oxidation and/or cleavage of the carbon-sulfur bonds of the organosulfur molecules by the biocatalyst, and (3) separating the fossil fuel having a reduced organic sulfur content from the resulting aqueous
20 phase.

The invention also provides a method of oxidizing an organic compound. The method comprises the steps of: (1) contacting the organic compound with an aqueous phase containing a *Sphingomonas* biocatalyst comprising at least
25 one enzyme capable of catalyzing at least one step in the oxidative cleavage of carbon-sulfur bonds, thereby forming an organic compound and aqueous phase mixture; (2) maintaining the mixture of step (1) under conditions sufficient for oxidation of the organic compound by the
30 biocatalyst, thereby resulting in an oxidized organic compound, and, optionally, separating the oxidized organic compound from the aqueous phase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, 1C and 1D together set forth the DNA sequence and the corresponding amino acid sequence of open reading frame 1 (ORF-1, *dszA*) of the nucleotide sequence
5 required for desulfurization activity in *Sphingomonas* sp. strain AD109.

Figures 2A, 2B and 2C together set forth the DNA sequence and the corresponding amino acid sequence of open reading frame 2 (ORF-2, *dszB*) of the nucleotide sequence
10 required for desulfurization activity in *Sphingomonas* sp. strain AD109.

Figures 3A, 3B and 3C together set forth the DNA sequence and the corresponding amino acid sequence of open reading frame 3 (ORF-3, *dszC*) of the nucleotide sequence
15 required for desulfurization activity in *Sphingomonas* sp. strain AD109.

Figure 4 is a graph showing the disappearance of 2-(2-phenyl)benzenesulfinate (HPBS) and the appearance of 2-hydroxybiphenyl (2-HBP) in the presence of *Sphingomonas*
20 AD109 cell-free lysates.

Figure 5 shows a physical map of the *Sphingomonas dsz* gene cluster.

Figures 6A, 6B, 6C, 6D, 6E, 6F and 6G together set forth the nucleotide sequence of the *Sphingomonas dsz* gene
25 cluster.

Figure 7 is a physical map of the plasmid pDA296.

Figure 8 presents the results of a GAP analysis of the DszA proteins from *Sphingomonas* sp. strain AD109 and *Rhodococcus* IGTS8.

30 Figure 9 presents the results of a GAP analysis of the DszB proteins from *Sphingomonas* sp. strain AD109 and *Rhodococcus* IGTS8.

Figure 10 presents the results of a GAP analysis of the sequences of the DszC proteins from *Sphingomonas* sp. strain AD109 and *Rhodococcus* IGTS8.

Figure 11 is a physical map of the plasmid pEBctac.

5 DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery and isolation of a novel microorganism which is capable of selectively desulfurizing dibenzothiophene ("DBT"). As described in Example 1, this microorganism was obtained from soil samples obtained at sites contaminated with petroleum and petroleum by-products by a soil enrichment procedure using 2-(2-hydroxyphenyl)benzenesulfinate as the sole sulfur source. A biologically pure sample of the novel microorganism has been isolated and characterized.

15 The microorganism is a motile, gram-negative rod. Based on a fatty acid analysis, as described in Example 2, this microorganism has been identified as a *Sphingomonas* species, and designated strain AD-109. This microorganism has been deposited at the American Type Culture Collection (ATCC), 12301 Park Lawn Drive, Rockville, Maryland, U.S.A. 20852 under the terms of the Budapest Treaty and has been designated as ATCC Deposit No. 55954 on April 21, 1997.

The novel microorganism of the invention can be grown by fermentation under aerobic conditions in the presence of a sulfur-free mineral salts medium (e.g., 4 g/L K_2HPO_4 , 4 g/L Na_2HPO_4 , 2 g/L NH_4Cl , 0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.001 g/L $CaCl_2 \cdot 2H_2O$, and 0.001 g/L $FeCl_3 \cdot 6H_2O$), containing a sulfur-free source of assimilable carbon such as glucose. The sole source of sulfur provided can be a heterocyclic organosulfur compound, such as dibenzothiophene or a derivative thereof.

Sphingomonas sp. strain AD109 expresses a collection of enzymes which together catalyze the conversion of DBT to

2-hydroxybiphenyl (also referred to as "2-HBP") and inorganic sulfur. An enzyme which catalyzes one or more steps in this overall process is referred to herein as a "desulfurization enzyme". The nucleic acid sequence
5 required for this overall process has been identified and cloned using the general method described in U.S. Patent No. 5,356,801, the contents of which are incorporated herein by reference, and is set forth in Figure 6 (SEQ ID NO.: 12). This nucleic acid sequence (also referred to as
10 the "*Sphingomonas* dsz sequence") comprises three open reading frames, designated ORF-1 (base pairs 442-1800, also set forth in Figures 1A-1D and SEQ ID NO.: 1), ORF-2 (base pairs 1800-2909, also set forth in Figures 2A-2C and SEQ ID NO.: 3) and ORF-3 (base pairs 2906-4141, sequence also set
15 forth in Figures 3A-3C and SEQ ID NO.: 5). The predicted amino acid sequences encoded by these open reading frames are set forth in Figures 1A-1D (ORF-1, SEQ ID NO.: 2), Figures 2A-2C (ORF-2, SEQ ID NO.: 4) and Figures 3A-3C (ORF-3, SEQ ID NO.: 6). Each of these open reading frames
20 is homologous to the corresponding open reading frame of *Rhodococcus* sp. IGTS8; the sequences of the *Rhodococcus* open reading frames are disclosed in U.S. Patent No. 5,356,801.

In one embodiment, the present invention provides an
25 isolated nucleic acid molecule comprising one or more nucleotide sequences which encode one or more of the biodesulfurization enzymes of *Sphingomonas* sp. strain AD109. The isolated nucleic acid molecule can be, for example, a nucleotide sequence, such as a deoxyribonucleic
30 acid (DNA) sequence or a ribonucleic acid (RNA) sequence. Such a nucleic acid molecule comprises one or more nucleotide sequences which encode one or more of the amino acid sequences set forth in SEQ ID NO.: 2, SEQ ID NO.: 4, and SEQ ID NO.: 6. For example, the isolated nucleic acid

molecule can comprise one or more of the nucleotide sequences of SEQ ID NO.: 1, SEQ ID NO.: 3, and SEQ ID NO.: 5, or a complement of any of these sequences. The isolated nucleic acid molecule can also comprise a nucleotide

5 sequence which results from a silent mutation of one or more of the sequences set forth in SEQ ID NO.: 1, SEQ ID NO.: 3, and SEQ ID NO.: 5. Such a nucleotide sequence can result, for example, from a mutation of the native sequence in which one or more codons have been replaced with a

10 degenerate codon, i.e., a codon which encodes the same amino acid. Such mutant nucleotide sequences can be constructed using methods which are well known in the art, for example the methods discussed by Ausubel *et al.*, *Current Protocols in Molecular Biology*, Wiley-Interscience,

15 New York (1997) (hereinafter "Ausubel *et al.*") and by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, third edition, Cold Spring Harbor Laboratory Press (1992) (hereinafter "Sambrook *et al.*"), each of which are incorporated herein by reference.

20 In another embodiment, the invention includes an isolated nucleic acid molecule comprising a nucleotide sequence which is homologous to one or more of the sequences of SEQ ID NO.: 1, SEQ ID NO.: 3, and SEQ ID NO.: 5, or complements thereof. Such a nucleotide sequence

25 exhibits at least about 80% homology, or sequence identity, with one of these *Sphingomonas* nucleotide sequences, preferably at least about 90% homology or sequence identity. Particularly preferred sequences have at least about 95% homology or have essentially the same sequence.

30 Preparation of mutant nucleotide sequences can be accomplished by methods known in the art as are described in Old, *et al.*, *Principles of Gene Manipulation*, Fourth

Edition, Blackwell Scientific Publications (1989), in Sambrook et al., and in Ausubel et al.

The invention further includes nucleic acid molecules which are useful as hybridization probes, for example, for the isolation of the *Sphingomonas* genes encoding desulfurization enzymes or identical or homologous genes from other organisms. Such molecules comprise nucleotide sequences which hybridize to all or a portion of the nucleotide sequence of SEQ ID NO.: 1, SEQ ID NO.: 3 or SEQ ID NO.: 5 or to non-coding regions immediately (within about 1000 nucleotides) 5' or 3' of each open reading frame. The invention also includes an isolated nucleic acid molecule which comprises a fragment of one or more of the nucleotide sequences set forth in SEQ ID NO.: 1, SEQ ID NO.: 3 or SEQ ID NO.: 5 or complements of any of these sequences. Such a fragment will generally comprise at least about 20 or at least about 40 contiguous nucleotides and, preferably, at least about 50 contiguous nucleotides of one of the disclosed sequences. Preferably, the hybridization probe of the invention hybridizes to one of these sequences under stringent conditions, such as those set forth by Sambrook et al. and Ausubel et al. For example, under conditions of high stringency, such as high temperatures and low salt concentrations, only DNA molecules which are essentially exact matches, or complements, will hybridize, particularly if the probe is relatively short. Hybridization under conditions of lower stringency, such as low temperatures, low formamide concentrations and high salt concentrations, allows greater mismatch between the probe and the target DNA molecule. It is particularly preferred that the nucleic acid molecule hybridizes selectively to the disclosed sequence(s).

The nucleic acid molecules can be synthesized chemically from the disclosed sequences. Alternatively,

the nucleic acid molecules can be isolated from a suitable nucleic acid library (such as a DNA library) obtained from a microorganism which is believed to possess the nucleic acid molecule (such as, *Sphingomonas* sp. strain AD109),
5 employing hybridizing primers and/or probes designed from the disclosed sequences. Such a method can result in isolating the disclosed molecules (or spontaneous mutants thereof) for use in preparing recombinant enzymes, confirming the disclosed sequences, or for use in
10 mutagenizing the native sequences.

In yet another embodiment, the nucleic acid molecule of the present invention can be a nucleic acid molecule, such as a recombinant DNA molecule, resulting from the insertion into its chain by chemical or biological means,
15 of one or more of the nucleotide sequences described above. Recombinant DNA includes any DNA synthesized by procedures using restriction nucleases, nucleic acid hybridization, DNA cloning, DNA synthesis or any combination of the preceding. Methods of construction can be found in
20 Sambrook et al. and Ausubel et al., and additional methods are known by those skilled in the art.

The isolated nucleic acid molecule of the invention can further comprise a nucleotide sequence which encodes an oxidoreductase, such as a flavoprotein, such as a flavin
25 reductase. For example, the nucleic acid molecule can encode an oxidoreductase which is native to *Sphingomonas* sp. strain AD109. The nucleic acid molecule can also encode the oxidoreductase denoted DszD described in copending U.S. Patent Application Serial No. 08/583,118;
30 the flavin reductase from *Vibrio harveyi* described in copending U.S. Patent Application Serial No. 08/351,754; or the flavin reductase from *Rhodococcus* sp. IGTS8, described in copending U.S. Patent Application Serial No. 08/735,963.

The contents of each of these applications are incorporated herein by reference.

The invention also includes a plasmid or vector comprising a recombinant DNA sequence or molecule which
5 comprises one or more of the nucleic acid molecules, e.g. nucleotide sequences, of the invention, as described above. The terms "plasmid" and "vector" are intended to encompass any replication competent or replication incompetent
10 plasmid or vector capable of having foreign or exogenous DNA inserted into it by chemical or biological means and subsequently, when transformed into an appropriate non-human host organism, of expressing the product of the foreign or exogenous DNA insert (e.g., of expressing the biocatalyst and flavoprotein of the present invention). In
15 addition, the plasmid or vector is receptive to the insertion of a DNA molecule or fragment thereof containing the gene or genes of the present invention, said gene or genes encoding a biocatalyst as described herein. Procedures for the construction of DNA plasmid vectors
20 include those described in Sambrook *et al.* and Ausubel *et al.* and others known by those skilled in the art.

The plasmids of the present invention include any DNA fragment containing a nucleotide sequence as described above. The DNA fragment should be transmittable, for
25 example, to a host microorganism by transformation or conjugation. Procedures for the construction or extraction of DNA plasmids include those described in Sambrook *et al.* and Ausubel *et al.*, and others known by those skilled in the art. In one embodiment, the plasmid comprises a
30 nucleotide sequence of the invention operatively linked to a competent or functional regulatory sequence. Examples of suitable regulatory sequences include promoters, enhancers, transcription binding sites, ribosomal binding sites, transcription termination sequences, etc.

In one preferred embodiment, the regulatory or promoter sequences are those native to the *Sphingomonas* operon containing the genes disclosed herein. In yet another embodiment, one or more regulatory sequences (e.g. the promoter) is native to the selected host cell for expression. The promoter can be selected so that the gene or genes are inducible or constitutively expressed. Furthermore, the sequences can be regulated individually or together, as an operon. Examples of suitable promoters include the *E. coli lac* and *tac* promoters and the Pseudomonas P_G promoter (Yen, *J. Bacteriol.* **173** : 5328-5335 (1991)). An example of such a plasmid and its construction are described in Example 8.

In another embodiment, the invention relates to a recombinant or transformed non-human host organism which contains a heterologous DNA molecule of the invention as described above. The recombinant non-human host organisms of the present invention can be created by various methods by those skilled in the art. Any method for introducing a recombinant plasmid, such as a plasmid of the invention described above, into the organism of choice can be used, and a variety of such methods are described by Sambrook et al. and Ausubel et al. For example, the recombinant plasmid can be introduced via a suitable vector by transformation, conjugation, transduction or electroporation. By the term "non-human host organism" is intended any non-human organism capable of the uptake and expression of foreign, exogenous or recombinant DNA.

The recombinant microorganism can be derived from a host organism which does not express a native desulfurization biocatalyst. Such microorganisms include bacteria and yeasts, e.g., *E. coli*, *Bacillus*, and non-desulfurizing pseudomonads (as described in U. S. Patent

Application Serial Number _____ (Attorney Docket No. EBC96-06A, being filed concurrently herewith)). In another embodiment, the recombinant microorganism is derived from a host organism which expresses a native biodesulfurization catalyst. Preferred microorganisms of this type are *Rhodococcus* sp. IGTS8 (ATCC 53968), recombinant microorganisms comprising one or more of the IGTS8 desulfurizing genes and *Sphingomonas* sp. strain AD109. Other desulfurizing microorganisms which are suitable host organisms include *Corynebacterium* sp. strain SY1, as disclosed by Omori et al., *Appl. Env. Microbiol.*, **58** : 911-915 (1992); *Rhodococcus erythropolis* D-1, as disclosed by Izumi et al., *Appl. Env. Microbiol.*, **60** :223-226 (1994); the *Arthrobacter* strain described by Lee et al., *Appl. Environ. Microbiol.* **61** : 4362-4366 (1995); the *Agrobacterium* strain disclosed by Constanti et al., *Enzyme Microb. Tech.* **19** : 214-219 (1996) and the *Rhodococcus* strains (ATCC 55309 and ATCC 55310) disclosed by Grossman et al., U.S. Patent No. 5,607,857, each of which is incorporated herein by reference in its entirety. Each of these microorganisms produces one or more enzymes (protein biocatalysts) that catalyze one or more reactions in the desulfurization of DBT.

The invention also relates to desulfurization enzymes which can be isolated from *Sphingomonas* sp. strain AD109. These include desulfurization enzymes which catalyze one or more steps in the oxidative desulfurization of DBT. The enzyme encoded by ORF-2 has been partially purified and exhibits 2-(2-hydroxyphenyl)benzenesulfinate (HPBS) desulfinase activity and has an apparent molecular weight by denaturing gel electrophoresis of about 40,000 daltons.

In one embodiment, the invention includes an isolated desulfurization enzyme from *Sphingomonas* sp. strain AD109 using methods and assays which are known the art, for example, the methods used by Gray et al. to isolate and
5 characterize desulfurization enzymes from *Rhodococcus* IGTS8 (Gray et al., *Nature Biotech.* **14** : 1705-1709 (1996)).

These enzymes can be isolated or purified from the cell by lysing the cell and subjecting the cell lysate to known protein purification methods, and testing the fractions
10 obtained thereby for the desired enzymatic activity. Examples of suitable protein purification methods include ammonium sulfate precipitation, ultrafiltration, diafiltration, immunoabsorption, anion exchange chromatography, gel filtration chromatography and
15 hydrophobic interaction chromatography. The enzymes of the invention can also be recombinant proteins produced by heterologous expression of a nucleic acid molecule comprising a nucleotide sequence of SEQ ID NO.: 1, SEQ ID NO.: 3 or SEQ ID NO.: 5; or a mutation or fragment thereof,
20 as discussed above. When the recombinant organism is derived from a non-*Sphingomonas* host, the recombinant proteins can be prepared in a form which is substantially free of other *Sphingomonas* proteins.

The invention also includes an isolated enzyme having
25 an amino acid sequence which is homologous to the amino acid sequence of SEQ ID NO.: 2, SEQ ID NO.: 4 or SEQ ID NO.: 6, or fragments thereof. The term "homologous" or "homologue", as used herein, describes a protein (which is not obtained from *Rhodococcus* or *Rhodococcus* sp IGTS8)
30 having at least about 80% sequence identity or homology with the reference protein, and preferably about 90% sequence homology, in an amino acid alignment. Most preferably, the protein exhibits at least about 95%

homology or essentially the same sequence as the disclosed sequence. An amino acid alignment of two or more proteins can be produced by methods known in the art, for example, using a suitable computer program, such as BLAST (Altschul
5 *et al.*, *J. Mol. Biol.* **215** : 403-410 (1990)). A homologous protein can also have one or more additional amino acids appended at the carboxyl terminus or amino terminus, such as a fusion protein.

The homologous enzymes described herein can be native
10 to an organism, such as a desulfurizing microorganism, including *Sphingomonas* sp. strain AD109 and mutants thereof. Such enzymes can be isolated from such sources using standard techniques and assays, as are described in the Exemplification and others known in the art. For
15 example, the *Sphingomonas* desulfurization enzymes can be used to induce the formation of antibodies, such as monoclonal antibodies, according to known methods. The antibodies can then be used to purify the desulfurization enzymes from a desulfurizing organism via affinity
20 chromatography, as is well known in the art.

The homologous enzymes of the invention can also be non-naturally occurring. For example, a homologous enzyme can be a mutant desulfurization enzyme which has a modified amino acid sequence resulting from the deletion, insertion
25 or substitution of one or more amino acid residues in the amino acid sequence of a *Sphingomonas* desulfurization enzyme. Such amino acid sequence variants can be prepared by methods known in the art. For example, the desired polypeptide can be synthesized *in vitro* using known methods
30 of peptide synthesis. The amino acid sequence variants are preferably made by introducing appropriate nucleotide changes into a DNA molecule encoding the native enzyme, followed by expression of the mutant enzyme in an

appropriate vector, such as *E. coli*. These methods include site-directed mutagenesis or random mutagenesis, for example.

Particularly preferred mutants include those having
5 amino acid sequences which include the amino acid residues which are encoded by both SEQ ID NO.: 1, SEQ ID NO.: 3 or SEQ ID NO.: 5 and the corresponding open reading frame of *Rhodococcus* sp. IGTS8, as disclosed in U.S. Patent No. 5,356,801. That is, these mutants include the amino acid
10 residues which are conserved in these two organisms in an amino acid alignment. Mutants which result from conservative substitution of one or more of these conserved residues, as well as non-conserved residues, are also included. Conservative and non-conservative substitutions
15 (including deletions and insertions) can be made in non-conserved regions of the amino acid sequence and mutants resulting from both conservative and non-conservative substitutions of these residues are included herein.

Conservative substitutions are those in which a first
20 amino acid residue is substituted by a second residue having similar side chain properties. An example of such a conservative substitution is replacement of one hydrophobic residue, such as valine, with another hydrophobic residue, such as leucine. A non-conservative substitution involves
25 replacing a first residue with a second residue having different side chain properties. An example of this type of substitution is the replacement of a hydrophobic residue, such as valine, with an acidic residue, such as glutamic acid.

30 The two primary variables in the construction of amino acid sequence variants are (1) the location of the mutation site and (2) the nature of the mutation. These variables can be manipulated to identify amino acid residues at the active site of the enzyme. For example, an amino acid

substitution which yields a mutant enzyme having significantly different activity than the native enzyme suggests that the substituted amino acid residue is at the active site. Such mutants can have the same or similar, increased or decreased activity relative to that of the native enzyme.

Amino acids can be modified, for example, by substituting first with a conservative choice, followed by non-conservative choices depending upon the results achieved, by deleting the target residue(s) or by inserting residues adjacent to a particular site. Variants can also be constructed using a combination of these approaches.

The proteins of the present invention can be produced using techniques to overexpress the gene, as are described by Sambrook et al. and Ausubel et al. Improved expression, activity or overexpression of the *Sphingomonas* desulfurization enzymes (in *Sphingomonas* sp AD 109 or in recombinant host cells harboring the disclosed nucleic acid molecules) can also be accomplished by mutagenesis. Suitable mutagens include radiation, e.g., ultraviolet radiation, and chemical mutagens, such as N-methyl-N'-nitroso-guanidine, hydroxylamine, ethylmethanesulfonate and nitrous acid. Furthermore, spontaneous mutants can be selected where the microorganism is subjected to an enrichment culture, as exemplified herein. The mutagenesis and subsequent screening for mutants harboring increased enzymatic activity can be conducted according to methods generally known in the art.

The present invention also provides a method of desulfurizing a carbonaceous material containing organosulfur molecules. The carbonaceous material can be, for example, a DBT-containing material or a fossil fuel, such as petroleum, a petroleum distillate fraction or coal. The method comprises the steps of (1) contacting the

carbonaceous material with an aqueous phase containing a *Sphingomonas*-derived biocatalyst comprising at least one enzyme capable of catalyzing at least one step in the oxidative cleavage of carbon-sulfur bonds, thereby forming
5 a carbonaceous material and aqueous phase mixture; (2) maintaining the mixture of step (1) under conditions sufficient for biocatalysis; and (3) separating the carbonaceous material having a reduced organic sulfur content from the resulting aqueous phase.

10 The term "*Sphingomonas*-derived biocatalyst", as used herein, is a biocatalyst which includes one or more desulfurization enzymes encoded by SEQ ID NO.: 1, SEQ ID NO.: 3 and SEQ ID NO.: 5; or a mutant or homologue thereof.

In one embodiment, the biocatalyst is a microorganism,
15 such as *Sphingomonas sp.* strain AD109. The biocatalyst can also be a recombinant organism which contains one or more heterologous nucleotide sequences or nucleic acid molecules as described above.

Although living microorganisms (e.g., a culture) can
20 be used as the biocatalyst herein, this is not required. Biocatalytic enzyme preparations that are useful in the present invention include microbial lysates, extracts, fractions, subfractions, or purified products obtained by conventional means and capable of carrying out the desired
25 biocatalytic function. Generally, such enzyme preparations are substantially free of intact microbial cells. In a particularly preferred embodiment, the biocatalyst is overexpressed in the recombinant host cell (such as a cell which contains more than one copy of the gene or genes).

30 Enzyme biocatalyst preparations suitable for use herein can optionally be affixed to a solid support, e.g., a membrane, filter, polymeric resin, glass particles or beads, or ceramic particles or beads. The use of immobilized enzyme preparations facilitates the separation

of the biocatalyst from the treated fossil fuel which has been depleted of refractory organosulfur compounds.

A fossil fuel that is suitable for desulfurization treatment according to the present invention is one that
5 contains organic sulfur. Such a fossil fuel is referred to as a "substrate fossil fuel". Substrate fossil fuels that are rich in thiophenic sulfur are particularly suitable for desulfurization according to the method described herein. Examples of such substrate fossil fuels include Cerro Negro
10 or Orinoco heavy crude oils; Athabaskan tar and other types of bitumen; petroleum refining fractions such as gasoline, kerosene, diesel, fuel oil, residual oils and miscellaneous refinery by-products; shale oil and shale oil fractions; and coal-derived liquids manufactured from sources such as
15 Pocahontas #3, Lewis-Stock, Australian Glencoe or Wyodak coal.

In the petroleum extraction and refining arts, the term "organic sulfur" is generally understood as referring to organic molecules having a hydrocarbon framework to
20 which one or more sulfur atoms are covalently joined. These sulfur atoms can be directly bonded to the hydrocarbon framework, e.g., by one or more carbon-sulfur bonds, or can be present in a substituent bonded to the hydrocarbon framework of the molecule, e.g., a sulfate
25 group. The general class of organic molecules having one or more sulfur heteroatoms are sometimes referred to as "organosulfur compounds". The hydrocarbon portion of these compounds can be aliphatic and/or aromatic.

Sulfur-bearing heterocycles, such as substituted and
30 unsubstituted thiophene, benzothiophene, and dibenzothiophene, are known to be stable to conventional desulfurization treatments, such as hydrodesulfurization (HDS). Sulfur-bearing heterocycles can have relatively simple or relatively complex chemical structures. In
35 complex heterocycles, multiple condensed aromatic rings,

one or more of which can be heterocyclic, are present. The difficulty of desulfurization generally increases with the structural complexity of the molecule. That is, refractory behavior is particularly accentuated in complex sulfur-bearing heterocycles, such as dibenzothiophene (DBT, $C_{12}H_8S$).

Much of the residual post-HDS organic sulfur in fossil fuel refining intermediates and combustible products is thiophenic sulfur. The majority of this residual thiophenic sulfur is present in DBT and derivatives thereof having one or more alkyl or aryl groups attached to one or more carbon atoms present in one or both flanking benzo rings. DBT itself is accepted as a model compound illustrative of the behavior of the class of compounds encompassing DBT and derivatives thereof in reactions involving thiophenic sulfur (Monticello and Finnerty, *Ann. Rev. Microbiol.*, **39** : 371-389 (1985)). DBT and derivatives thereof can account for a significant percentage of the total sulfur content of particular crude oils, coals and bitumen. For example, these sulfur-bearing heterocycles have been reported to account for as much as 70 wt% of the total sulfur content of West Texas crude oil, and up to 40 wt% of the total sulfur content of some Middle East crude oils. Thus, DBT is considered to be particularly relevant as a model compound for the forms of thiophenic sulfur found in fossil fuels, such as crude oils, coals or bitumen of particular geographic origin, and various refining intermediates and fuel products manufactured therefrom (Monticello and Finnerty (1985), *supra*). Another characteristic of DBT and derivatives thereof is that, following a release of fossil fuel into the environment, these sulfur-bearing heterocycles persist for long periods of time without significant biodegradation. Gundlach et al., *Science* **221** : 122-129 (1983). Thus, most prevalent naturally

occurring microorganisms do not effectively metabolize and break down sulfur-bearing heterocycles.

Biocatalytic desulfurization (biocatalysis or BDS) is the excision (liberation or removal) of sulfur from
5 organosulfur compounds, including refractory organosulfur compounds such as sulfur-bearing heterocycles, as a result of the oxidative, preferably selective, cleavage of carbon-sulfur bonds in said compounds by a biocatalyst. BDS treatment yields the desulfurized combustible hydrocarbon
10 framework of the former refractory organosulfur compound, along with inorganic sulfur substances which can be readily separated from each other by known techniques such as fractional distillation or water extraction. For example, DBT is converted into 2-hydroxybiphenyl when subjected to
15 BDS treatment. A suitable biocatalyst for BDS comprises *Sphingomonas* sp. strain AD109 or an enzyme preparation derived therefrom, optionally, in combination with one or more additional non-human desulfurizing organisms (e.g., microorganisms); or an enzyme preparation derived from such
20 an organism. Suitable additional desulfurizing organisms include those described above.

The specific activity of a given biocatalyst is a measure of its biocatalytic activity per unit mass. Thus, the specific activity of a particular biocatalyst depends
25 on the nature or identity of the microorganism used or used as a source of biocatalytic enzymes, as well as the procedures used for preparing and/or storing the biocatalyst preparation. The concentration of a particular biocatalyst can be adjusted as desired for use in particular circum-
30 stances. For example, where a culture of living microorganisms, such as *Sphingomonas* sp. strain AD109, is used as the biocatalyst preparation, a suitable culture medium lacking a sulfur source other than sulfur-bearing heterocycles can be inoculated with suitable microorganisms

and grown until a desired culture density is reached. The resulting culture can be diluted with additional medium or another suitable buffer, or microbial cells present in the culture can be retrieved e.g., by centrifugation, and
5 resuspended at a greater concentration than that of the original culture. The concentrations of microorganism and enzyme biocatalyst can be adjusted similarly. In this manner, appropriate volumes of biocatalyst preparations having predetermined specific activities and/or concentra-
10 tions can be obtained.

In the biocatalytic desulfurization stage, the liquid fossil fuel containing sulfur-bearing heterocycles is combined with the biocatalyst. The relative amounts of biocatalyst and liquid fossil fuel can be adjusted to suit
15 particular conditions, or to produce a particular level of residual sulfur in the treated, deeply desulfurized fossil fuel. The amount of biocatalyst preparation to be combined with a given quantity of liquid fossil fuel will reflect the nature, concentration and specific activity of the
20 particular biocatalyst used, as well as the nature and relative abundance of inorganic and organic sulfur compounds present in the substrate fossil fuel and the degree of deep desulfurization sought or considered acceptable.

25 The method of desulfurizing a fossil fuel of the present invention involves two aspects. First, a host organism or biocatalytic preparation obtained therefrom is contacted with a fossil fuel to be desulfurized. This can be done in any appropriate container, optionally fitted
30 with an agitation or mixing device. The mixture is combined thoroughly and maintained or allowed to incubate for a sufficient time to allow for biocatalysis. In one embodiment, an aqueous emulsion or microemulsion is produced with an aqueous culture of the organism or enzyme
35 fraction and the fossil fuel, allowing the organism to

propagate in the emulsion while the expressed biocatalyst cleaves carbon-sulfur bonds.

Variables such as temperature, pH, oxidation levels, mixing rate and rate of desulfurization will vary according to the nature of the biocatalyst used. Optimal parameters can generally be determined through no more than routine experimentation.

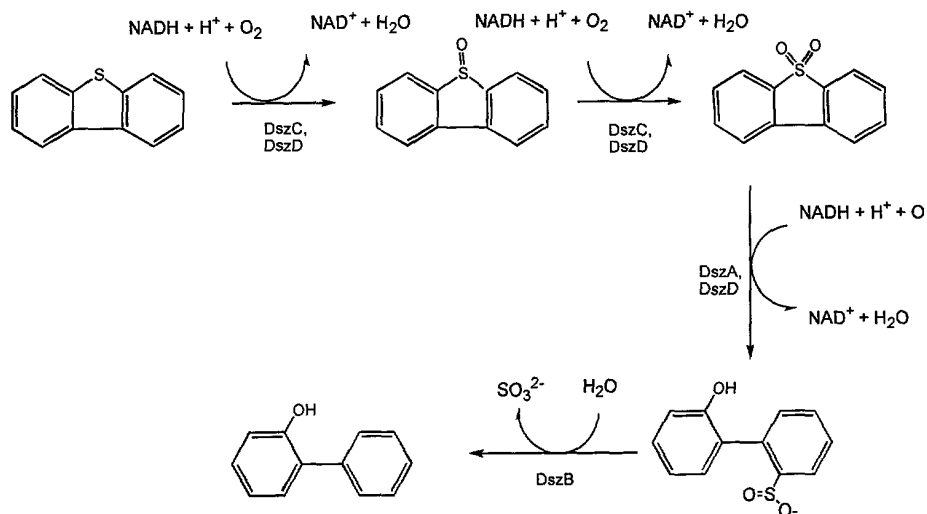
When the fossil fuel is a liquid hydrocarbon, such as petroleum, the desulfurized fossil fuel and the aqueous phase can form an emulsion. The components of such emulsions can be separated by a variety of methods, such as those described in U.S. Patent No. 5,358,870 and U.S. Patent Application Serial No. 08/640,129, which are incorporated herein by reference. For example, some emulsions reverse spontaneously when maintained under stationary conditions for a suitable period of time. Other emulsions can be reversed by adding an additional amount of an aqueous phase. Still other emulsions can be separated by the addition of a suitable chemical agent, such as a demulsifying agent or by employing suitable physical conditions, such as a particular temperature range.

The biocatalyst can be recovered from the aqueous phase, for example, by centrifugation, filtration or lyophilization. When the biocatalyst is a microorganism, the biocatalyst can be resuspended in fresh sulfur-free nutrient medium and/or any fresh microorganism culture as necessary to reconstitute or replenish to the desired level of biocatalytic activity. The biocatalyst can then be reintroduced into the reaction system.

Several suitable techniques for monitoring the rate and extent of desulfurization are well-known and readily available to those skilled in the art. Baseline and time course samples can be collected from the incubation mixture, and prepared for a determination of the residual organic sulfur in the fossil fuel. The disappearance of

sulfur from organosulfur compounds, such as DBT, in the sample being subjected to biocatalytic treatment can be monitored using, e.g., X-ray fluorescence (XRF) or atomic emission spectrometry (flame spectrometry). Preferably, the molecular components of the sample are first separated, e.g., by gas chromatography.

Without being limited to any particular mechanism or theory, it is believed that the pathway of the desulfurization reaction in *Spingomonas* sp. strain AD109 and other desulfurizing organisms, such as *Rhodococcus* sp. IGTS8, is set forth below:



Here the flavin reductase provides an electron transport chain which delivers, via FMNH₂, the reducing equivalents from NADH (or other electron donor) to the enzymes DszC and/or DszA. The enzyme DszC is responsible for the biocatalysis of the oxidation reaction of DBT to DBTO₂. The enzyme DszA is responsible for the reaction of DBTO₂ to

2-(2-hydroxyphenyl)benzenesulfinate (HPBS). The enzyme DszB catalyzes the conversion of HPBS to 2-hydroxybiphenyl and inorganic sulfur.

Another method of use of the *Sphingomonas*

5 desulfurization enzymes, or mutants, homologues or active fragments thereof, is as a biocatalyst for the oxidation of organic compounds, such as substituted or unsubstituted dibenzothiophenes. The method comprises the steps of (1) contacting the organic compound with an aqueous phase
10 containing a *Sphingomonas*-derived biocatalyst comprising at least one enzyme capable of catalyzing at least one step in the oxidative cleavage of carbon-sulfur bonds, thereby forming an organic compound and aqueous phase mixture; (2) maintaining the mixture of step (1) under conditions
15 sufficient for oxidation of the organic compound by the biocatalyst, thereby resulting in an oxidized organic compound, and, optionally, separating the oxidized organic compound from the aqueous phase. In one embodiment, the organic compound is a heteroorganic compound, such as an
20 organonitrogen compound or an organosulfur compound. In one embodiment, the organic compound is an organosulfur compound which is a component of a fossil fuel, such as petroleum or a petroleum distillate fraction. In a second embodiment, the organic compound is a substituted or
25 unsubstituted indole, as described in U.S. Provisional Patent Application Serial Number 60/020563, filed July 2, 1996, which is incorporated herein by reference.

The enzyme encoded by the nucleotide sequence of ORF-3 catalyzes the oxidation of dibenzothiophene to
30 dibenzothiophene-5,5-dioxide (dibenzothiophene sulfone), and the enzyme encoded by the nucleotide sequence of ORF-1 catalyzes the oxidation of dibenzothiophene-5,5-dioxide to 2-(2-hydroxyphenyl)benzenesulfinate (also referred to as "HPBS"). In one embodiment the biocatalyst comprises the

enzyme encoded by ORF-3, or a mutant, homologue or active fragment thereof; the organosulfur compound is substituted or unsubstituted dibenzothiophene; and the oxidized organosulfur is a substituted or unsubstituted

5 dibenzothiophene-5,5-dioxide or dibenzothiophene-5-oxide (dibenzothiophene sulfoxide). In another embodiment the biocatalyst comprises the enzymes encoded by ORF-1 and ORF-3, or a mutant, homologue or active fragment thereof; the organosulfur compound is a substituted or unsubstituted

10 dibenzothiophene; and the oxidized organosulfur compound is a substituted or unsubstituted 2-(2-hydroxyphenyl)benzenesulfinate. In yet another embodiment, the biocatalyst comprises the enzyme encoded by ORF-1 or a mutant, homologue or active fragment thereof; the

15 organosulfur compound is a substituted or unsubstituted dibenzothiophene-5,5-dioxide; and the oxidized organosulfur compound is a substituted or unsubstituted 2-(2-hydroxyphenyl)benzenesulfinate.

The oxidized organosulfur compound can, optionally,

20 be further processed, for example, via a non-biological process or an enzyme-catalyzed reaction. In one embodiment, the oxidized organosulfur compound is desulfurized in a process employing suitable desulfurization enzymes from an organism other than a

25 *Sphingomonas*.

The biocatalyst can be an organism, such as *Sphingomonas* sp. strain AD109, a desulfurizing mutant thereof, or a recombinant organism or enzyme preparation, as discussed above. When the organosulfur compound is a

30 component of a fossil fuel, suitable reaction conditions and fossil fuel sources can be determined as described above.

The invention will now be further illustrated by the way of the following examples.

EXAMPLES

General Methods and Materials

Bacterial strains and plasmids

E. coli DH10 β (F⁻ *mcrA*

- 5 $\Delta(mrr-hsdRMS-mcrBC)\phi$ 80dlacZ Δ M15 Δ lacX74 *deoR recA1 endA1*
ara Δ 139 $\Delta(ara, leu)$ 7697 *galU galK lambda⁻ rpsL nupG*;
Gibco-BRL, Gaithersburg, MD) was used as the cloning host.
Plasmids pUC18 (Ap^R; Viera and Messing, Gene 19 :
259-268, (1982)), pOK12 (Km^R; Viera and Messing, Gene 100
10 : 189-194 (1991)) and pSL1180 (Ap^R; Brosius, DNA 8 : 759,
(1989)) were used as cloning vectors. Plasmid pEBCTac (Ap^R
Tc^R *lacI^q tac*, shown in Figure 11, was used to
overexpress the *Sphingomonas dszB* in *E. coli*.

Media and Reagents

- 15 Luria broth (LB) medium was routinely used to
propagate *E. coli*. LB medium is 1% tryptone (Difco), 0.5%
yeast extract (Difco) and 0.5% NaCl. Rich medium (RM) was
used to propagate *Sphingomonas* strain AD109. RM medium is
0.8% nutrient broth, 0.05% yeast extract and 1% glucose.
20 2YT medium, used in gene expression studies, is 1.6%
tryptone, 1% yeast extract and 0.5% NaCl. Basal salts
medium (BSM-glucose) contained the following (per liter):
phosphate buffer 100 mmol (pH 7.2); glucose, 20 g; NH₄Cl, 2
g; MgCl₂·6H₂O, 644 mg; MnCl₂·4H₂O, 1 mg; nitriloacetic acid,
25 0.1 g; FeCl₂·4H₂O, 2.6 mg; Na₂B₄O₇·10H₂O, 0.1 mg; CuCl₂·2H₂O,
0.15 mg; Co(NO₃)₂·6H₂O, 0.125 mg; ZnCl₂, 2.6 mg; CaCl₂·2H₂O,
33 mg; (NH₄)₆Mo₇O₂₄·4H₂O, 0.09 mg; and EDTA, 1.25 mg. When
required the sulfur source was either 2 mM MgSO₄, 300 μ M
Dibenzothiophene (DBT), 300 μ M Dibenzothiophene sulfone

(DBTO₂) or 300 μ M 2-(2-hydroxyphenyl) benzenesulfinate (HPBS). For solid media, agar or agarose was added at a concentration of 1.5% (wt/wt). The antibiotic concentrations for *E. coli* were as follows: ampicillin, 100 μ g/ml; kanamycin, 30 μ g/ml; tetracycline, 10 μ g/ml.

DNA Methods

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA) and used as recommended by the supplier. Chromosomal DNA was isolated by the method described by Woo et al., *BioTechniques* **13**: 696-698 (1992). Small scale plasmid preparations from *E. coli* were carried out as described by Birboim and Doly, *Nuc. Acids Res.* **7** : 1513-1523 (1979). Larger scale DNA preparations were carried out with Midi-prep columns from Qiagen (Chatsworth, CA). DNA fragments were purified from agarose gels after electrophoretic separation by the method of Vogelstein and Gillespie (*Proc. Natl. Acad. Sci. USA* **76**: 615-619 (1979). DNA fragments were cloned into vectors by using techniques described by Sambrook et al.

Degenerate oligonucleotide probes were end-labeled using standard digoxigenin protocols according to the Boehringer Mannheim DIG Oligonucleotide 3'-End Labeling Kit (Cat. No. 1362372). Hybridization was performed in 5X SSC with blocking solution containing 50% ultrapure deionized formamide at 42°C overnight (16 hr). Detection of hybrids was by enzyme immunoassay according to the Boehringer Mannheim Nonradioactive DIG DNA Labeling and Detection Kit (Cat. No. 1093657).

DNA samples were sequenced by SeqWright (Houston, TX) using a dye-terminator cycling sequencing kit from Perkin Elmer and the 373A and 377 ABI automatic DNA sequencer. The sequence was extended by synthesizing overlapping

oligonucleotides to previously read sequence. The synthesized oligonucleotides were used as primers for continuing sequence reactions. Sequencing reads were assembled and edited to 99.99% accuracy using Genecode's
5 *Sequencher*, version 3.0 computer software.

DNA and protein sequence analysis was performed with the MacVector software program (Oxford Molecular Group, Campbell, CA). Nucleotide and amino acid sequences were compared to sequences in the available databases using
10 BLAST. The Wisconsin Genetics Computer Group (GCG) software (Devereux *et al.*, *Nucl. Acids Res.* **12** : 387-395 (1984)) program GAP was used to generate comparisons of the protein sequences.

15 Transformation of *E. coli*

Plasmid DNA was introduced into *E. coli* DH10 β by electroporation. Competent ElectroMAX DH10 β (Gibco-BRL, Gaithersburg, MD) were used according to the manufacturer's suggestions.

20 Preparation of cell-free extracts

Cells grown in the appropriate medium were concentrated to an optical density at 600 nm of 50 by centrifugation and resuspended in 10 mM phosphate buffer (pH 7.0). Cells were disrupted in a French press and
25 debris was removed by centrifugation at 32,000 x g for 20 min. Cell lysates were stored on ice at 4°C.

Desulfurization assays and analytical analysis

HPBS desulfinase activity was assayed by the ability of cell-free lysates to convert HPBS (substrate) to 2-HBP
30 (product) in a one hour assay at 30°C. The amounts of product made and substrate consumed during the reaction

were quantitated by high-pressure liquid chromatography (HPLC) analysis. HPBS desulfhinase activity was also measured by fluorescence spectroscopy. In a typical enzyme assay, enzyme activity is determined by the change in
5 fluorescence at an excitation wavelength of 288 nm and an emission wavelength of 414 nm as HPBS is converted to 2-HBP. The assay is initiated by the addition of 20 - 100 μ g total protein to a 3 mL solution of 200 μ M HPBS in 50 mM phosphate buffer pH 7.5 containing 0.1 M NaCl.

10 Expression studies

E. coli DH10 β harboring the *Sphingomonas dszB* overexpression plasmid pDA296 was inoculated into 100ml of 2YT medium containing ampicillin and allowed to grow with shaking at 30°C. At an OD₆₀₀ of approximately 0.3, the
15 culture was divided into two parts. One half of the culture was induced by the addition of isopropylthio- β -galactoside (IPTG) (final conc. 1 mM) and the remaining culture was used as an uninduced control (no IPTG was added). Following incubation for an additional 3 hr, both
20 cultures were harvested and cell-free lysates were prepared.

Protein purification and N-terminal sequencing

Sphingomonas AD109 cell paste was resuspended in an approximately equal weight of 25 mM phosphate buffer pH 7.5
25 containing 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 10 μ g/mL DNase and 1 mM phenylmethylsulfonyl fluoride and passed through a French press mini-cell at about 20,000 psi. Cell debris was removed by centrifugation and the cell lysate was fractionated over an Econo-Pac High Q
30 cartridge manufactured by Bio-Rad. A linear 0-0.5 M NaCl gradient was used to elute the bound protein into

fractions. The active fractions were identified by a 2-HBP fluorescence enzyme assay (excitation/emission wavelengths set at 288/414 nm). The active fractions were pooled and desalted over a Bio-Rad P6 gel filtration cartridge, 5 diluted to 1.7 M ammonium sulfate and fractionated over a Phenyl Superose HR 5/5 column manufactured by Pharmacia. A linear 1.7-0.0 M ammonium sulfate gradient was used to elute protein into fractions. Active fractions were identified and pooled as described above. Identity and 10 purity of the AD109 HPBS desulfinate protein was also determined by SDS-PAGE and Western blots using antibodies generated against the DszB protein from *Rhodococcus erythropolis* strain IGTS8. N-terminal microsequencing of the HPBS desulfinate was carried out by Edman degradation 15 after transfer of the purified protein to a polyvinylidene difluoride (PVDF) membrane.

SDS-PAGE and Western Blot Analysis

Protein separations were done with Novex (San Diego, CA) precast 10% polyacrylamide gels with Tris-Glycine- 20 sodium dodecyl sulfate (SDS) (Laemmli) running buffer. Western blot analysis was carried out by first transferring the proteins electrophoretically to nitrocellulose membranes as recommended by Biorad (Hercules, CA). Blots were treated with antisera raised against the purified 25 IGTS8 DszB protein (primary antibody) and then with goat anti-rabbit antisera conjugated to horseradish peroxidase as the secondary antibody. Finally, the proteins were detected with a horseradish peroxidase catalyzed chemiluminescent reaction.

Example 1 Soil enrichments and isolation of a
 microorganism that can use HPBS as a sole
 sulfur source

5 Three independent soil samples from oil-contaminated
 sites were used to perform soil enrichments for
 microorganisms able to use HPBS as a sole sulfur source.
 Approximately 5 grams of each soil sample was placed into a
 sterile 250 ml flask along with 50 ml of BSM Glucose medium
10 containing HPBS (300 μ M) as the sole source of sulfur.
 Following incubation for 96 hrs at 30°C, a 3 ml sample of
 each enrichment was transferred to fresh BSM Glucose medium
 containing HPBS. After 72 hrs, one of the three flasks
 (flask #3) showed visible turbidity, while the two
15 remaining flasks showed no visible increase in turbidity
 (even after more than a week of incubation). Microscopic
 analysis of the contents of flask #3 revealed the presence
 of a mixed population of bacterial cells (i.e., sessile and
 motile rods of varying shapes; large and small coccoid
20 shaped bacteria). After repeated liquid subculture
 enrichments with HPBS as the sole sulfur source, the
 contents of the flask was plated onto several RM and LB
 agar plates. Following incubation at 30°C, a variety of
 microorganisms with different colony morphologies was
25 present. Analysis of individual colonies from these plates
 identified a pure isolate that efficiently used HPBS as a
 sole sulfur source. This strain, designated AD109, was
 selected for further analysis.

Example 2 Characterization and identification of
30 strain AD109

 The HPBS utilizing strain AD109 is a Gram-negative,
 motile rod that forms distinctive yellow colonies on agar
 plates. It grows somewhat poorly on LB agar, but grows

rather well on RM agar plates. Like *Rhodococcus* IGTS8, strain AD109 also has the ability to produce clearing zones on a BSM Glucose DBT-sulfone plate. The optimal growth temperature of AD109 was found to be between 30 and 37°C.

5 Based on fatty acid analysis (Acculab, Inc., Newark, DE), this strain was identified as a *Sphingomonas* species. Strain AD109 was a "good" match to *S. paucimobilis* (formerly *Pseudomonas paucimobilis*) based on its "similarity index". The similarity index is a mathematical
10 expression of the extent to which the fatty acid profile of a given unknown matches the mean profile for an organism in the TSBA database. Strain AD109 had an index value of 0.426 which indicates that it is from a strain of a species that differs significantly from those represented in the
15 database. A similarity index of 0.5 or above is considered to be an "excellent" match (a value of 1.0 being the highest possible). On the other hand, an index below 0.3 indicates that the sample is from a species that is not likely to be in the database. Based on 16S rRNA sequence
20 analysis and the presence of sphingoglycolipids, Yabuuchi *et al.* (*Microbiol. Immunol.* **34** : 99-119 (1990)) proposed that *P. paucimobilis* be reclassified and placed into the genus *Sphingomonas*.

25 Example 3 Growth characteristics of *Sphingomonas*
 species strain AD109

Evidence for the existence of an HPBS desulfinate activity was demonstrated by monitoring the supernatant of a AD109 culture growing in BSM Glucose HPBS (300 µM). By
30 the time the culture was well into stationary phase all of the HPBS had been converted with no apparent accumulation of identifiable intermediates. There was, however, a

transient production of a small amount of 2-HBP, as determined by HPLC analysis, which also disappeared with time. This preliminary result suggested that AD109 may also be capable of metabolizing 2-HBP. *Sphingomonas* strain

5 AD109 was also capable of utilizing DBT-sulfone (DBTO₂) as a sole sulfur source. The ability to utilize DBT-sulfone as a sole sulfur source suggests that strain AD109 may also contain a gene that encodes DBT-sulfone monooxygenase activity.

10 During the course of growth studies it was discovered that strain AD109 could utilize DBT as a sole sulfur source. While growing with DBT, however, the culture supernatant takes on a very characteristic orange/brown color with an absorption maximum of approximately 470 nm.
15 Orange-colored oxidation products have been previously identified in a number of *Pseudomonas* species that are capable of degrading DBT (Monticello et al., *Appl. Environ. Microbiol.* **49** : 756-760 (1985)); Foght and Westlake, *Can. J. Microbiol.* **36** : 718-724 (1990)). No such color
20 development was detected in cultures growing with either HPBS or DBT-sulfone as sulfur sources.

Example 4 Demonstration of HPBS desulfinase activity
 in AD109 cell-free lysates

25 A cell-free lysate prepared from a culture of *Sphingomonas* strain AD109 (grown in BSM Glucose medium containing HPBS) was used in a time course study to examine the rate at which HPBS is converted to 2-HBP. As presented in Figure 4, at a protein concentration of 4 mg/ml there was a linear increase in 2-HBP production and a concomitant
30 disappearance of HPBS.

The product of the *in vitro* reaction was confirmed to be 2-HBP by a spectral comparison to authentic 2-HBP. The ultraviolet absorption spectrum of the suspected 2-HBP peak produced by the action of the AD109 lysate is virtually
5 identical with that of the 2-HBP standard. Furthermore, the molecular weight of the unknown compound was exactly that of authentic 2-HBP as determined by GC-MS analysis.

Example 5 Purification of the HPBS desulfinate from
Sphingomonas AD109

10 HPBS desulfinate was purified from AD109 by a series of chromatographic steps using a Bio-Rad low pressure column chromatography Econo system and a Pharmacia FPLC (Gray et al., *Nature Biotech.* **14** : 1705-1709 (1996)). The steps included fractionation over an anion exchange resin
15 followed by a hydrophobic interaction column chromatography step. These protein purification steps are described above. A 15-20 fold purification was achieved in these two steps which is comparable to protein preparations from a *Rhodococcus* IGTS8 lysate.

20 The molecular weight of this protein by SDS-PAGE was estimated to be 40,000 daltons, which is approximately the same size as DszB purified from IGTS8. Western analysis demonstrated that the purified protein shows some cross-reactivity with anti-DszB antisera.

25 Nonlinear regression analysis of an enzyme progress curve was performed according to the general method described by Duggleby, *Methods Enzymol.* **249** : 61-90 (1995). The analysis involves fitting the integrated Michealis-Menton rate equation $V_m^*t = y - K_m^* \ln(1 - y/[A]_0)$ to
30 concentration vs. time data from the enzyme catalyzed reaction of 2-(2-phenyl)benzenesulfinate to 2-

hydroxybiphenyl monitored to completion by fluorescence. The semi-pure protein sample was generated by fractionation of a crude lysate over Q Sepharose Fast Flow resin (Pharmacia) by a linear 0-0.5 M NaCl gradient, as discussed
5 in more detail above. The purity of the active fraction was determined by SDS-PAGE. Pure enzyme is not necessary for the application of enzyme progress curve analysis, however, the calculation of k_{cat} ($V_m = [E]_t * k_{cat}$) was limited to a value range as only a crude estimate of the enzyme
10 concentration was available. The reaction conditions were as follows. A 3 mL reaction solution containing 1 μ M HPBS and 0.1 M NaCl in 50 mM phosphate at pH 7.5 and 30°C was initiated by the addition of 0.023 mg total protein and was monitored for 30 min by fluorescence at an excitation
15 wavelength of 288 nm and an emission wavelength of 414 nm. The data were fit to the equation using the Kaleidagraph data analysis/graphics application (Abelleck Software).

Based on the kinetic parameters calculated from the enzyme progress assay ($K_m=0.3 \mu$ M and $V_m=0.1 \mu$ M/min), the
20 minimum $k_{cat} = 0.5 \text{ min}^{-1}$. However, a more realistic value would be on the order of 2 min^{-1} in view of the fact that the preparation is estimated to be about 25% pure. Therefore, the HPBS desulfonase from *Sphingomonas* AD109 appears to be comparable to that from *Rhodococcus* IGTS8
25 with the possibility of a higher catalytic efficiency (k_{cat}/K_m).

The N-terminal amino acid sequence of the purified *Sphingomonas* HPBS desulfonase was also determined. Protein microsequencing using standard methods of analysis resulted
30 in the following amino acid sequence:

1 10 20
TTDIHPASAA SSPAARATIT YS (SEQ ID NO.: 7)

A comparison of the putative AD109 HPBS desulfinase N-terminal sequence with that of the N-terminus of the IGTS8 DszB protein revealed that 9 out the 22 amino acid residues were identical (41%). In order to determine whether the purified protein is, in fact, the *Sphingomonas* desulfinase protein, a degenerate (192 permutations) 17-mer oligonucleotide probe with the following sequence: 5' ACN GAY ATH CAY CCN GC 3' (SEQ ID NO.: 8), was designed based on the determined N-terminal sequence. Following labeling with a non-isotopic label this probe was used in hybridization studies using the cloned *Sphingomonas* AD109 HPBS desulfinase gene (see below) and the *dszB* gene from IGTS8 (Denome et al., *J. Bacteriol.* **176** : 6707-6716 (1994); Piddington et al., *App. Environ. Microbiol.* **61** : 468-475 (1995). The labeled oligonucleotide probe hybridized to the cloned *Sphingomonas* HPBS desulfinase gene which indicated that the correct protein had been purified. However, no signal was detected in the lane containing a fragment harboring the *Rhodococcus dsz B* gene.

Example 6 Cloning of the *Sphingomonas* AD109 HPBS desulfinase gene

Strain AD109 has been shown to be capable of using HPBS as a sole sulfur source and clearing a DBTO₂ plate. On the assumption that the gene(s) responsible for DBTO₂ clearing and HPBS desulfinase activity are genetically closely linked, as they are in *Rhodococcus* IGTS8, a cloning scheme was devised to isolate the HPBS desulfinase gene from *Sphingomonas* strain AD109. Total genomic DNA from strain AD109 was digested with either *EcoRI*, *BamHI*, and *HindIII* and the resulting fragments were ligated into pUC18

or pSL1180. Following transformation of *E. coli* DH10 β , approximately 1000-2000 Lac-negative, ampicillin-resistant colonies of each library were screened for the ability to clear a DBTO₂ plate. No clearing colonies were detected
5 amongst transformants derived from either the *Eco*RI or *Bam*HI libraries. However, two clearing colonies were detected utilizing the *Hind*III library and one clearing colony was detected with the *Not*I library. Based on restriction endonuclease profiles, both colonies from the
10 *Hind*III library contained the same large fragment (~20 kb). Furthermore, there was measurable HPBS desulfinate activity in cell-free lysates of these strains.

The single clearing colony from the *Not*I library contained a 6.5 kb fragment which, according to restriction
15 endonuclease mapping, overlapped the 20 kb *Hind*III fragment. This clone also contained measurable HPBS desulfinate activity.

Subcloning analysis localized the genes responsible for DBTO₂ clearing and HPBS desulfinate activity to a 6 kb
20 *Hind*III-*Not*I fragment. A smaller 2.7 kb *Hind*III-*Sma*I fragment was subsequently found to retain HPBS desulfinate activity, but lost the ability to clear a DBTO₂ plate. It is likely, therefore, that the gene that confers the ability to produce clearing zones on a DBT-sulfone plate
25 spans the *Sma*I site.

Example 7 DNA sequence analysis of the *Sphingomonas*
 sp. strain AD109 desulfurization gene
 cluster

The nucleotide sequence of a 4144 bp region which
30 encompasses the AD109 HPBS desulfinate gene was

determined from both DNA strands and is present in Figure 6 (SEQ ID NO.: 12). The overall G+C content of the first 3837 base pairs of the AD109 sequence is 64.5%, a value which is consistent with the range of G+C values (61.7 - 67.2%) reported for various *Sphingomonas* species (Yabuuchi et al. (1990)). A comparison of the AD109 nucleotide sequence with the IGTS8 *dsz* sequence by DNA matrix analysis revealed that a considerable amount of homology exists between the two sequences as evidenced by the presence of a near continuous diagonal line.

Open reading frame analysis of the AD109 sequence revealed the presence of a number of ORFs on both DNA strands, but of these, only three contained the codon-choice pattern characteristic of microorganisms with G-C rich genomes (West et al., Nucl. Acids Res. 16: 9323-9334 (1988)). All three identified ORFs were in the same transcriptional orientation. A strong preference for codons with either G or C occurred in positions 1 and 3. The first codon position of all three ORFs ranged from 67 to 72%, while the third codon position of all three ORFs ranged from 79-81%. In addition, the predicted translation initiation sites of all three ORFs are preceded by sequences that resemble a consensus ribosome binding site.

The entire nucleotide sequence of the AD109 region was used to conduct a BLAST search of the available DNA databases. The *Rhodococcus* IGTS8 *dsz* genes were the highest scoring sequences that demonstrated homology to the *Sphingomonas* sequences. The only other nucleotide sequence that demonstrated any significant homology to the *Sphingomonas* DNA, was the *Streptomyces pristinaespiralis* *snaA* gene which encodes the large

subunit of the PII_A synthase (Blanc et al., J. Bacteriol. 177 : 5206-5214 (1995)). The *Sphingomonas dszA* and *S. pristinaespiralis snaA* genes demonstrate about 60% identity over a 800 bp region proximal to the 5' end of each gene.

The first ORF (bp 442-1800; Figures 1A-1D) is 71% identical (at the nucleotide level) to the *Rhodococcus dszA* gene. The primary translation product of ORF-1 would encode a protein (*Sphingomonas* DszA or Dsz(S)) that contains 453 amino acids with a predicted molecular weight of 50,200. More importantly, this protein demonstrates considerable homology to the amino acid sequence of *Rhodococcus* DszA (Dsz(R), SEQ ID NO.: 9) over the entire length of the polypeptide (76% identity and 87% similarity; Figure 8). The protein databases were also searched with the *Sphingomonas* DszA protein sequences. Aside from the DszA protein of *Rhodococcus* IGTS8, several other proteins demonstrated significant homology to the *Sphingomonas* DszA protein. These include a hypothetical 49.3 kD protein in the IDH-DEOR intergenic region of *Bacillus subtilis* which showed 45% identity over 382 residues, the PII_A synthase SnaA subunit of *S. pristinaespiralis* (Blanc et al., J. Bacteriol. 177 : 5206-5214 (1995)) which was 49% identical over 358 residues and the nitrilotriacetate monooxygenase of *Chelatobacter heintzii* (Xu et al., Abstracts of the 95th General Meeting of the American Society for Microbiology, Q-281) which was 50% identical over the 335 residues examined.

The stop site of the *Sphingomonas* ORF-1 shows a 4-bp overlap with the translation start site of the second ORF (bp 1800-2906; Figures 2A-2C), which shows a

high degree of homology to the *Rhodococcus* IGTS8 *dszB* gene (67% identity). It was determined that the primary translation product of ORF-2 would encode a 369-amino acid polypeptide with a predicted molecular weight of
5 40,000 (*Sphingomonas* DszB or Dsz(S)). At the amino acid level this putative protein is 66% identical (75% similarity) to the *Rhodococcus* HPBS desulfinase protein DszB (DszB(R), SEQ ID NO: 10), as shown in Figure 9. Except for the IGTS8 DszB protein, a BLAST search with
10 the *Sphingomonas* DszB sequence did not identify any other significant homologous sequences in the available databases. The predicted N-terminus of the *Sphingomonas* DszB protein matches identically the N-terminus of the HPBS desulfinase purified from AD109 cell lysates,
15 except that the amino-terminal methionine was absent. Removal of the methionine residue has been shown to occur when the second amino acid is Ala, Ser, Gly, Pro, Thr or Val (Hirel et al., *Proc. Nat. Acad. Sci. USA* **86** : 8247-8251 (1989)).

20 The stop site of the *Sphingomonas dszB* gene also shows a 4-bp overlap with the translation start site of the third ORF. This ORF (bp 2906-4141; Figures 3A-3C), shows significant homology to the *Rhodococcus* IGTS8 *dszC* gene. For example, over the first 931 bp, this ORF is
25 69% identical to the IGTS8 *dszC* gene and the N-terminus of the protein predicted by this sequence (*Sphingomonas* DszC, DszC(S)) is 67% identical to the N-terminus of *Rhodococcus* DszC (DszC(R), SEQ ID NO: 11), as shown in Figure 10. A BLAST search of the protein databases with
30 the available *Sphingomonas* DszC sequence identified a number of proteins in addition to the IGTS8 DszC protein. The *Sphingomonas* DszC protein is 32% identical

(over 199 residues) to Isobutylamine N-Hydroxylase (IBAH) of *Streptomyces viridifaciens*. It has previously been shown that IBAH exhibits the greatest similarity to the IGTS8 DszC protein (Parry et al., *J. Bacteriol.*,

5 179: 409-416 (1997)). In addition, the AD109 DszC protein showed variable homology to a number of acyl coenzyme A dehydrogenases. For example, the N-terminal 300 residues of the *Sphingomonas* DszC protein is 29% identical to the acyl CoA dehydrogenase of *B. subtilis*.

10 The sequences (400 bp) directly upstream of the *dszA* start site contain regulatory elements (i.e., promoter elements) that control transcription of the AD109 *dsz* gene cluster. A comparison of this potential promoter region with the IGTS8 *dsz* promoter region
15 failed to reveal any significant homology. It has been shown that the IGTS8 *dsz* promoter region encompasses a region of potential diad symmetry that may contain an operator (Li et al., *J. Bacteriol.* 178 : 6409-6418 (1996)). An examination of the AD109 sequences directly
20 upstream of *dszA* revealed no such palindromic sequence.

Example 8 Expression of the *Sphingomonas dszB* gene in *E. coli*

The AD109 *dszB* gene was subcloned into the *tac* promoter expression vector, pEBCTac, in two steps. The
25 first step involved cloning a 1.2 kb *Pst*I-*Bgl*II fragment that contained the entire coding region of the AD109 *dszB* gene (Figures 2A-2C) into the polylinker plasmid pOK12. The resulting plasmid, designated pDA295, contained a unique *Xba*I site upstream of the *dszB* gene.

In the second step, a 1.2 kb *Xba*I-*Bgl*III fragment from pDA295 that contained the entire *dszB* gene was cloned into the *Xba*I and *Bgl*III sites of pEBCTac, thus placing the AD109 *dszB* gene under the transcriptional control of the *tac* promoter. This plasmid, designated pDA296 and presented in Figure 7, was introduced into *E. coli* DH10 β for expression studies.

HPBS desulfinate assays (2 mg/ml protein) using cell-free lysates prepared from induced and uninduced cultures of DH10 β /pDA296 were performed. In the absence of IPTG the cell-free lysate contained very little HPBS desulfinate activity. Only 22 nmoles of 2-HBP were produced during the 60 min. incubation period which corresponds to a specific activity of 0.2 (nmoles 2-HBP formed/min/mg protein). The lysate prepared from the IPTG-induced culture, however, had approximately 20 times more HPBS desulfinate activity (4.2 nmoles 2-HBP formed/min/mg protein) than the lysate prepared from the uninduced culture.

20 EQUIVALENTS

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A culture of *Sphingomonas* sp. strain AD109 or a mutant thereof.
- 5 2. A nucleotide molecule encoding an enzyme having an amino acid sequence set forth in SEQ ID NO.: 2; or a mutant, fragment or homologue thereof.
3. The nucleotide molecule of Claim 2 having substantially the same sequence as the sequence set
10 forth in SEQ ID NO.: 1.
4. A nucleotide molecule encoding an enzyme having an amino acid sequence set forth in SEQ ID NO.: 4; or a mutant, fragment or homologue thereof.
5. The nucleotide molecule of Claim 4 having substantially the same sequence set forth in SEQ ID
15 NO.: 3.
6. A nucleotide molecule encoding an enzyme having an amino acid sequence set forth in SEQ ID NO.: 6; or a mutant, fragment or homologue thereof.
- 20 7. The nucleotide molecule of Claim 6 having substantially the same sequence as the sequence set forth in SEQ ID NO.: 5.
8. A nucleic acid molecule comprising the nucleotide
25 sequence set forth in SEQ ID NO.: 1, SEQ ID NO.: 3, or SEQ ID NO.: 5; a mutant or fragment thereof; or a combination thereof.

9. A nucleotide sequence comprising at least about 20 contiguous nucleotides from the sequence of SEQ ID NO.: 1, or the complement thereof.
- 5 10. The nucleotide sequence of Claim 9 comprising at least about 40 contiguous nucleotides from the sequence of SEQ ID NO.: 1 or the complement thereof.
- 10 11. The nucleotide sequence of Claim 9 comprising at least about 50 contiguous nucleotides from the sequence of SEQ ID NO.: 1 or the complement thereof.
12. A nucleotide sequence comprising at least about 20 contiguous nucleotides from the sequence of SEQ ID NO.: 3, or the complement thereof.
- 15 13. The nucleotide sequence of Claim 12 comprising at least about 40 contiguous nucleotides from the sequence of SEQ ID NO.: 3 or the complement thereof.
- 20 14. The nucleotide sequence of Claim 12 comprising at least about 50 contiguous nucleotides from the sequence of SEQ ID NO.: 3 or the complement thereof.
- 25 15. A nucleotide sequence comprising at least about 20 contiguous nucleotides from the sequence of SEQ ID NO.: 5, or the complement thereof.
16. The nucleotide sequence of Claim 15 comprising at least about 40 contiguous nucleotides.

17. The nucleotide sequence of Claim 15 comprising at least about 50 contiguous nucleotides.
18. A nucleotide sequence which specifically hybridizes to a polynucleotide molecule comprising the nucleotide sequence set forth in SEQ ID NO.: 1.
19. The nucleotide sequence of Claim 18, wherein the isolated nucleotide sequence hybridizes to the polynucleotide molecule under conditions of high stringency.
20. A nucleotide sequence which hybridizes to a polynucleotide molecule comprising the nucleotide sequence set forth in SEQ ID NO.: 3.
21. The nucleotide sequence of Claim 20, wherein the isolated nucleotide sequence hybridizes to the polynucleotide molecule under conditions of high stringency.
22. A nucleotide sequence which hybridizes to a polynucleotide molecule comprising the nucleotide sequence set forth in SEQ ID NO.: 5.
23. The nucleotide sequence of Claim 22, wherein the isolated nucleotide sequence hybridizes to the polynucleotide molecule under conditions of high stringency.
24. An enzyme having substantially the amino acid sequence set forth in SEQ ID NO.: 2, or an enzymatically active fragment thereof.

25. The enzyme of Claim 24, wherein said enzyme is isolated from a microorganism.
26. The enzyme of Claim 25 wherein the microorganism is a *Sphingomonas*.
- 5 27. The enzyme of Claim 26 wherein the microorganism is *Sphingomonas* sp. strain AD109.
28. The enzyme of Claim 27 having substantially the amino acid sequence set forth in SEQ ID NO.: 2, or fragment thereof, said enzyme being substantially
10 free of other *Sphingomonas* proteins.
29. An enzyme having substantially the amino acid sequence set forth in SEQ ID NO.: 4, or an enzymatically active fragment thereof.
30. The enzyme of Claim 29, wherein said enzyme is
15 isolated from a microorganism.
31. The enzyme of Claim 30 wherein the microorganism is a *Sphingomonas*.
32. The enzyme of Claim 31 wherein the microorganism is *Sphingomonas* sp. strain AD109.
- 20 33. The enzyme of Claim 32 having a molecular weight of about 40,000 daltons.

34. An enzyme having substantially the amino acid sequence set forth in SEQ ID NO.: 4, or fragment thereof, said enzyme being substantially free of other *Sphingomonas* proteins.
- 5 35. An enzyme having substantially the amino acid sequence set forth in SEQ ID NO.: 6, or an enzymatically active fragment thereof.
36. The enzyme of Claim 35, wherein said enzyme is isolated from a microorganism.
- 10 37. The enzyme of Claim 36 wherein the microorganism is a *Sphingomonas*.
38. The enzyme of Claim 37 wherein the microorganism is *Sphingomonas* sp. strain AD109.
- 15 39. An enzyme having substantially the amino acid sequence set forth in SEQ ID NO.: 6, or fragment thereof, said enzyme being substantially free of other *Sphingomonas* proteins.
40. An enzyme comprising the amino acid sequence set forth in SEQ ID NO.: 2.
- 20 41. An enzyme comprising the amino acid sequence set forth in SEQ ID NO.: 4.
42. An enzyme comprising the amino acid sequence set forth in SEQ ID NO.: 6.
- 25 43. A *Sphingomonas* enzyme catalyzing the conversion of dibenzothiophene to dibenzothiophene-5,5-dioxide.

44. A *Sphingomonas* enzyme catalyzing the conversion of dibenzothiophene-5,5-dioxide to 2-(2-hydroxyphenyl)benzenesulfinate.
45. A *Sphingomonas* enzyme catalyzing the conversion of
5 2-(2-hydroxyphenyl)benzenesulfinate to 2-hydroxybiphenyl and inorganic sulfur.
46. A plasmid comprising a nucleic acid molecule of Claim 2 operatively linked to a promoter.
- 10 47. A plasmid comprising a nucleic acid molecule of Claim 4 operatively linked to a promoter.
48. A plasmid comprising a nucleic acid molecule of Claim 6 operatively linked to a promoter.
49. A plasmid comprising a nucleotide molecule of Claim
15 8 operatively linked to a promoter.
50. A transformed microorganism containing a recombinant DNA plasmid comprising a DNA molecule encoding an enzyme set forth in SEQ ID NO.: 2, a mutant or a homologue thereof.
- 20 51. A transformed microorganism containing a recombinant DNA plasmid comprising a DNA molecule encoding an enzyme having the sequence set forth in SEQ ID NO.: 4, or a mutant, fragment or homologue thereof.

52. A transformed microorganism containing a recombinant DNA plasmid comprising a DNA molecule encoding an enzyme having the amino acid sequence set forth in SEQ ID NO.: 6; or a mutant or
5 homologue thereof.
53. A transformed microorganism containing a recombinant DNA plasmid comprising a DNA sequence encoding an enzyme having the sequence set forth in SEQ ID NO.: 2, or a mutant, fragment or homologue
10 thereof; a DNA sequence encoding an enzyme having the sequence set forth in SEQ ID NO.: 4, or a mutant, fragment or homologue thereof; and a DNA sequence encoding an enzyme having the amino acid sequence set forth in SEQ ID NO.: 6, or a mutant,
15 fragment or homologue thereof.
54. A method of desulfurizing a fossil fuel containing organosulfur molecules, comprising the steps of:
(a) contacting the fossil fuel with an aqueous phase containing a *Sphingomonas*-derived
20 desulfurization biocatalyst thereby forming a fossil fuel and aqueous phase mixture;
(b) maintaining the mixture under conditions sufficient for desulfurization, thereby
25 resulting in a fossil fuel having a reduced organic sulfur content; and
(c) separating the fossil fuel having a reduced organic sulfur content from the resulting aqueous phase.
55. The method of Claim 54 wherein the biocatalyst
30 comprises an enzyme preparation or a microorganism comprising an enzyme having the sequence set forth

in SEQ ID NO.: 2, or a mutant, active fragment or
homologue thereof; an enzyme having the sequence
set forth in SEQ ID NO.: 4, or a mutant, active
fragment or homologue thereof; and an enzyme having
5 the amino acid sequence set forth in SEQ ID NO.: 6,
or a mutant, active fragment or homologue thereof.

56. The method of Claim 55 further comprising the steps
of adding a flavoprotein, flavin, NADH or a
combination thereof.

10 57. The method of Claim 55 wherein the fossil fuel is a
liquid hydrocarbon.

58. The method of Claim 57 wherein the liquid
hydrocarbon is a petroleum.

15 59. The method of Claim 55 wherein the biocatalyst is a
microorganism.

60. The method of Claim 59 wherein the microorganism is
Sphingomonas sp. strain AD109.

20 61. The method of Claim 59 wherein the microorganism
contains a heterologous DNA molecule which encodes
the biocatalyst.

62. The method of Claim 61 wherein the heterologous DNA
is derived from *Sphingomonas* sp. strain AD109.

63. The method of Claim 55 wherein the biocatalyst is a
cell-free fraction.

64. The method of Claim 63 wherein the biocatalyst is a cell-free fraction of *Sphingomonas* sp. strain AD109.
65. A method of oxidizing organic molecules, comprising the steps of:
- (a) contacting the organic molecules with an aqueous phase containing a *Sphingomonas*-derived biocatalyst capable of oxidizing organosulfur compounds, thereby forming an organic compound and aqueous phase mixture; and
 - (b) maintaining the mixture under conditions sufficient for oxidation of the organic molecules by the biocatalyst, thereby forming an oxidized organic compound.
66. The method of Claim 65 wherein the biocatalyst comprises an enzyme having the sequence set forth in SEQ ID NO.: 2, or a mutant, active fragment or homologue thereof; an enzyme having the sequence set forth in SEQ ID NO.: 4, or a mutant, active fragment or homologue thereof; an enzyme having the amino acid sequence set forth in SEQ ID NO.: 6, or a mutant, active fragment or homologue thereof; or a combination thereof.
67. The method of Claim 66 wherein the organic is an organosulfur compound which is a component of a fossil fuel.

68. The method of Claim 67 wherein the organosulfur compound is a substituted or unsubstituted dibenzothiophene and the oxidized organosulfur compound is a substituted or unsubstituted dibenzothiophene-5-5-dioxide.
69. The method of Claim 67 wherein the organosulfur compound is a substituted or unsubstituted dibenzothiophene-5-5-dioxide and the oxidized organosulfur compound is a substituted or unsubstituted 2-(2-hydroxyphenyl)benzenesulfinate.
70. The method of Claim 66 wherein the biocatalyst capable of oxidizing organosulfur molecules is a microorganism.
71. The method of Claim 70 wherein the microorganism is *Sphingomonas* sp. strain AD109.
72. The method of Claim 70 wherein the microorganism contains a heterologous DNA molecule which encodes the biocatalyst.
73. The method of Claim 72 wherein the heterologous DNA is derived from *Sphingomonas* sp. strain AD109.
74. The method of Claim 66 wherein the biocatalyst capable of oxidizing organosulfur compounds is a cell-free fraction.

75. The method of Claim 74 wherein the biocatalyst is a cell-free fraction of *Sphingomonas* sp. strain AD109.

A *SPHINGOMONAS* BIODESULFURIZATION CATALYST

Abstract of the Disclosure

The invention relates to a novel microorganism, designated *Sphingomonas* sp. strain AD109, which is
5 capable of selectively desulfurizing dibenzothiophene. The invention also includes isolated proteins and nucleic acid sequences obtained from this microorganism.

In another embodiment, the invention provides a method of using this microorganism or enzyme preparations
10 derived therefrom in the biocatalytic desulfurization of a fossil fuel containing organic sulfur compounds.

[illegible]

FIGURE 1A

Sphingomonas ORF1 (cont)

450 *
 AGC AAT GCA GAG GCG CGC AAC TTC GGC TTC GAT GAA CAT CTC GAC CAC GAT GCC CGC TAC
 S N A E A R N F G F D E H L D H D A R Y>
 510 *
 GAT CGC GCC GAT GAA TTC CTC GAG GTC GTG CGC AAG CTC TGG AAC AGC TGG GAT CGC GAT
 D R A D E F L E V V R K L W N S W D R D>
 570 *
 GCG CTG ACA CTC GAC AAG GCA ACC GGC CAG TTC GCC GAT CCG GCT AAG GTG CGC TAC ATC
 A L T L D K A A T G Q F A D P A K V R Y I>
 630 *
 GAC CAC CGC GGC GAA TGG CTC AAC GTA CGC GGG CCG CTT CAG GTG CCG CGC TCC CCC CAG
 D H R G G E W L N V R G P L Q V P R S P Q>
 690 *
 GCG GAG CCT GTC ATT CTG CAG GCC GGC CTT TCG GCG CGG AAG CGC TTC GCC GGG CGC
 G E P V I L Q C A G L S A R G K R F A G R>
 750 *
 TGG GCG GAC GCG GTG TTC ACG ATT TCG CCG AAT CTG GAC ATC ATG CAG GCC ACG TAC CGC
 W A D A V F T I S P N L D I M Q A T Y R>
 810 *
 GAC ATA AAG GCG CAG GTC GAG GCC GGC GGA CGC GAT CCC GAG CAG GTC AAG GTG TTT GCC
 D I K A Q V E A A G R D P E Q V K V F A>

FIGURE 1B

Sphingomonas ORF1 (cont.)

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370 *
GCG GTG ATG CCG ATC CTC GGC GAG ACC GAG GCG ATC GCC AGG CAG CGT CTC GAA TAC ATA
A V M P I L G E T E A I A R Q R L E Y I>
900 *

930 *
AAT TCG CTG GTG CAT CCC GAA GTC GGG CTT TCT ACG TTG TCC AGC CAT GTC GGG GTC AAC
N S L V H P E V G L S T L S S H V G V N>
960 *

990 *
CTT GCC GAC TAT TCG CTC GAT ACC CCG CTG ACC GAG GTC CTG GGC GAT CTC GCC CAG CGC
L A D Y S L D T P L T E V L G D L A Q R>
1020 *

1050 *
AAC GTG CCC ACC CAA CTG GGC ATG TTC GCC AGG ATG TTG CAG GCC GAG ACG CTG ACC GTG
N V P T Q L G M F A R M L Q A E T L T V>
1080 *

1110 *
GGA GAA ATG GGC CCG CGT TAT GGC GCC AAC GTG GGC TTC GTC CCG CAG TGG GCG GGA ACC
G E M G R R Y G A N V G F V P Q W A G T>
1140 *

1170 *
CGC GAG CAG ATC GCG GAC CTG ATC GAG ATC CAT TTC AAG GCC GGC GGC GAT GGC TTC
R E Q I A D L I E I H F P K A G G A D G F>
1200 *

1230 *
ATC ATC TCG CCG GCG TTC CTG CCC GGA TCT TAC GAG GAA TTC GTC GAT CAG GTG GTG CCC
I I S P A F L CTG CCC GGA TCT TAC GAG GAA TTC GTC GAT CAG GTG GTG CCC
1260 *

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FIGURE 1C

[illegible]

FIGURE 1D

FIGURE 2A

FIGURE 2C

FIGURE 2C

Sphingomonas ORF3

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10  *      20  *      30  *      40  *      50  *      60  *
ATG AAC GAA CTC GTC AAA GAT CTC GGC CTC AAT CGA TCC GAT CCG ATC GGC GGT GTG CGG
M  N  E  L  V  K  D  L  G  L  N  R  S  D  P  I  G  A  V  R>

70  *      80  *      90  *      100 *      110 *      120 *
CGA CTG GCC GCG CAG TGG GGG GCC ACC GCT GTT GAT CGG GAC CCG GCC GGA TCG GCA
R  L  A  A  Q  W  G  A  T  A  V  D  R  D  R  A  G  G  S  A>

130 *      140 *      150 *      160 *      170 *      180 *
ACC GCC GAA CTC GAT CAA CTG CGC GGC AGC GGC CTG CTC TCG TCG TCC ATT CCC GCC GCA
T  A  E  L  D  Q  L  R  G  S  G  L  L  S  L  S  I  P  A  A>

190 *      200 *      210 *      220 *      230 *      240 *
TAT GGC GGC TGG GGC GCC GAC TGG CCA ACG ACT CTG GAA GTT ATC CGC GAA GTC GCA ACG
Y  G  G  W  G  A  D  W  P  T  T  L  E  V  I  R  E  V  A  T>

250 *      260 *      270 *      280 *      290 *      300 *
GTG GAC GGA TCG CTG GCG CAT CTA TTC GGC TAC CAC CTC GGC TGC GTA CCG ATG ATC GAG
V  D  G  S  L  A  H  L  F  G  Y  H  L  G  C  V  P  M  I  E>

310 *      320 *      330 *      340 *      350 *      360 *
CTG TTC GGC TCG GCG CCA CAA AAG GAA CCG CTG TAC CGC CAG ATC GCA AGC CAT GAT TGG
L  F  G  S  A  P  Q  K  E  R  L  Y  R  Q  I  A  S  H  D  W>

370 *      380 *      390 *      400 *      410 *      420 *
CGG GTC GGG AAT GCG TCG AGC GAA AAC AAC AGC CAC GTG CTC GAG TGG AAG CTT GCC GCC
R  V  G  N  A  S  S  E  N  N  S  H  V  L  E  W  K  L  A  A>

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FIGURE 3A

Sphingomonas ORF3 (cont.)

ACC GCC GTC GAT GAT GGC GGC TTC GTC CTC AAC GGC GCG AAG CAC TTC TGC AGC GGC GCC	430 *	440 *	450 *	460 *	470 *	480 *
T A V D D G G G G G F V L N G A K H F C S G A>						
AAA AGC TCC GAC CTG CTC ATC GTG TTC GGC GTG ATC CAG GAC GAA TCC CCC CTG CGC GGC	490 *	500 *	510 *	520 *	530 *	540 *
K S S D L L I V F G V I Q D E S P L R G>						
GCG ATC ATC ACC GCG GTC ATT CCC ACC GAC CGG GCC GGT GTT CAG ATC AAT GAC GAC TGG	550 *	560 *	570 *	580 *	590 *	600 *
A I I T A V I P T D R A G V Q I N D D W>						
CGC GCA ATC GGC ATG CGC CAG ACC GAC AGC GGC AGC GCC GAA TTT CGC GAC GTC CGA GTC	610 *	620 *	630 *	640 *	650 *	660 *
R A I G M R Q T D S G S A E F R D V R V>						
TAC CCA GAC GAG ATC TTG GGC GCA CCA AAC TCA GTC GTT GAG GCG TTC GTG ACA AGC AAC	670 *	680 *	690 *	700 *	710 *	720 *
Y P D E I L G A P N S V V E A F T S N>						
CGC GGC AGC CTG TGG ACG CCG GCG ATT CAG TCG ATC TTC TCG AAC GTT TAT CTG GGG CTC	730 *	740 *	750 *	760 *	770 *	780 *
R G S L W T P A I Q S I F S N V Y L G L>						
GCG CGT GGC GCG CTC GAG GCG GCA GCG GAT TAC ACC CGG ACC CAG AGC CGC CCC TGG ACA	790 *	800 *	810 *	820 *	830 *	840 *
A R G A L E A A A D Y T R T Q S R P W T>						

FIGURE 3B

Sphingomonas ORF3 (cont)

CCC GCC GGC GTG GCG AAG GCG ACA GAG GAT CCC CAC ATC ATC GGC ACC TAC GGT GAA CTG	850	860	870	880	890	900
P A G V A A K A T E D P H I I A T Y G E L>	*	*	*	*	*	*
GCG ATC GCG CTC CAG GGC GGC GAG GCG GCC GCG CAC GAG GTC GCG GCC CTG TTG CAA CAG	910	920	930	940	950	960
A I A L Q CAG GGC GGC GAG GCG GCC GCG CAC GAG GTC GCG GCC CTG TTG CAA CAG	*	*	*	*	*	*
GCG TGG GAC AAG GGC GAT GCG GTG ACG CCC GAA GAG CGC GGC CAG CTG ATG GTG AAG GTT	970	980	990	1000	1010	1020
A W D K G G D A A V T P E E R G Q L M V K V>	*	*	*	*	*	*
TCG GGT GTG AAG GCC CTC TCG ACG AAG GCC GGC GGC GGC GGC GGC GGC GGC GGC GGC GGC	1030	1040	1050	1060	1070	1080
S G V K A A L S T K A A A L D I T S R I F E>	*	*	*	*	*	*
ACA ACG GGC TCG CGA TCG ACG CAT CCC AGA TAC GGA TTC GAT CGG TTC TGG CGT AAC ATC	1090	1100	1110	1120	1130	1140
T T G S R S T H P R Y G F D D R N I>	*	*	*	*	*	*
CGG ACT CAT ACG CTG CAC GAT CCG GTA TCG TAT AAA ATC GTC GAT GTG GGC AAC TAC ACG	1150	1160	1170	1180	1190	1200
R T H T L L H D P V S Y K I V D V G N Y T>	*	*	*	*	*	*
CTC AAC GGC ACA TTC CCG GTT CCC GGA TTT ACG TCA	1210	1220	1230			
L N G T F P V P G F T S>	*	*	*			

FIGURE 3C

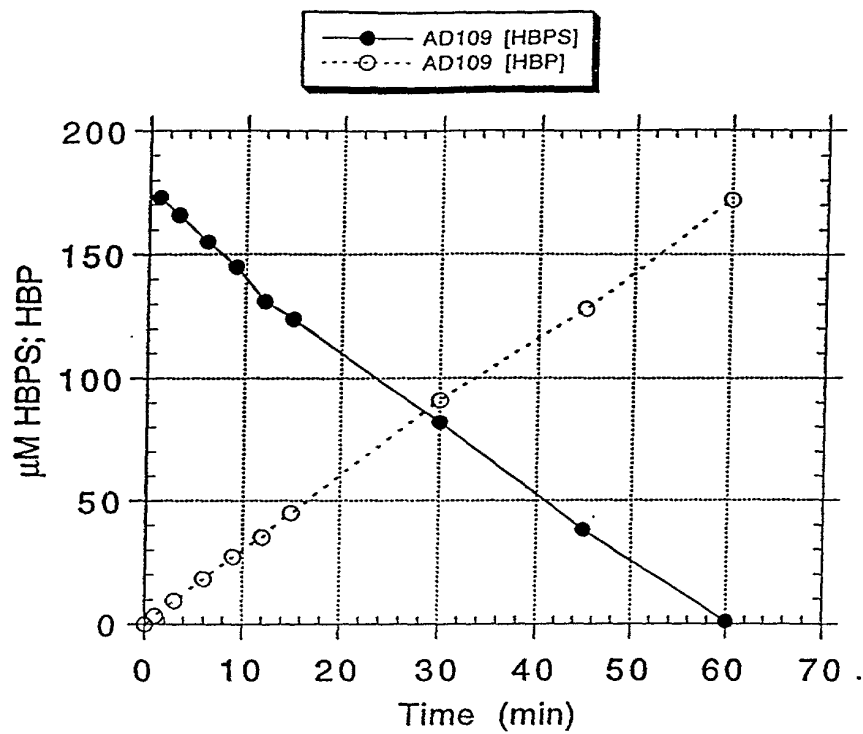


FIGURE 4

200 bp

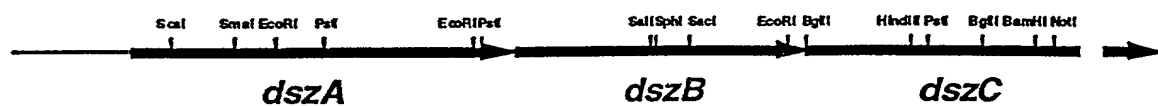


FIGURE 5

Sphingomonas dsz sequence

10	20	30	40	50	60
*	*	*	*	*	*
GGTTCGAGAT	CGATCTGACC	GTCTGAACCCG	GCGCGGTTCA	AACCATCCTC	TGGGGCCTCT
CCAAGCTCTA	GCTAGACTGG	CAGCTTGGGC	CGCGCCAAGT	TTGGTAGGAG	ACCCCGGAGA
70	80	90	100	110	120
*	*	*	*	*	*
TCTTGCACTT	GACATAGGAA	TCTCTACTAA	ATAAATAGAT	ATTTATTTCGA	CAC'TAAGTTC
AGAACGTGAA	CTGTATCCTT	AGAGATGATT	TATTTATCTA	TAAATAAGCT	GTGATTCAAG
130	140	150	160	170	180
*	*	*	*	*	*
GGTGATCAGG	CCGACCGTGT	GTCTCAAGTG	CTCGCTCCGG	GTTGCCACGA	GCTAAAGCGC
CCACTAGTCC	GGCTGGCACA	CAGAGTTCAC	GAGCGAGGCC	CAACGGTGCT	CGATTTCGCG
190	200	210	220	230	240
*	*	*	*	*	*
GCGATGCTGG	GGCGACAGCG	CTAGGCATTG	CGTTCCCTCA	CACCAATGAT	GAGATGATAC
CGCTACGACC	CCGCTGTGCG	GATCCGTAAC	GCAAGGGAGT	GTGGTTACTA	CTCTACTATG
250	260	270	280	290	300
*	*	*	*	*	*
GATGCGCATG	ACCACTATCC	GCACCTAGCA	CGAAAGATCC	GTGCATTTTCG	CGAATGCCAA
CTACGCGTAC	TGGTGATAGG	CGTGGATCGT	GCTTTCTAGG	CACGTAAAGC	GCTTACGGTT
310	320	330	340	350	360
*	*	*	*	*	*
TGAAGAGGAC	CGACGTACGG	CAGCTTCCTA	CGCTTTCGCG	CCATCGTTCA	TAGCCAAGGT
ACTTCTCCTG	GCTGCATGCC	GTCTGAAGGAT	GCGAAAGCGC	GGTAGCAAGT	ATCGGTTCCA
370	380	390	400	410	420
*	*	*	*	*	*
CTTTTCGACG	CCGGTTCGCG	TGGGCGACTG	ACGGCGGTAG	CGCCGCGACT	ATTTCGTTTCA
GAAAAGCTGC	GGCCAAGCGC	ACCCGCTGAC	TGCCGCCATC	GCGGCGCTGA	TAAGCAAAGT
430	440	450	460	470	480
*	*	*	*	*	*
AACTCACGAG	GATAAGAGCC	TATGACCGAT	CCACGTCAGC	TGCACCTGGC	CGGATTCTTC
TTGAGTGCTC	CTATTCTCGG	ATACTGGCTA	GGTGCAGTCG	ACGTGGACCG	GCCTAAGAAG
490	500	510	520	530	540
*	*	*	*	*	*
TGTGCCGGCA	ACGTCACGCA	CGCCCACGGA	GCGTGGCGCC	ACGCCGACGA	CTCCAACGGC
ACACGGCCGT	TGCAGTGCGT	GCGGGTGCCT	CGCACCGCGG	TGCGGCTGCT	GAGGTTGCCG
550	560	570	580	590	600
*	*	*	*	*	*
TTCTTCACCA	AGGAGTACTA	CCAGCAGATT	GCCCCGACGC	TCGAGCGCGG	CAAGTTCGAC
AAGGAGTGGT	TCCTCATGAT	GGTCGTCTAA	CGGGCGTGCG	AGCTCGCGCC	GTTCAAGCTG

FIGURE 6A

Sphingomonas dsz sequence (page 2)

610	620	630	640	650	660
*	*	*	*	*	*
CTGCTGTTCC	TTCCCGACGC	GCTCGCCGTG	TGGGACAGCT	ACGGCGACAA	TCTGGAGACC
GACGACAAGG	AAGGGCTGCG	CGAGCGGCAC	ACCCTGTCTGA	TGCCGCTGTT	AGACCTCTGG
670	680	690	700	710	720
*	*	*	*	*	*
GGTCTGCGGT	ATGGCGGGCA	AGGCGCGGTG	ATGCTGGAGC	CCGGCGTAGT	TATCGCCGCG
CCAGACGCCA	TACCGCCCGT	TCCGCGCCAC	TACGACCTCG	GGCCGCATCA	ATAGCGGCGC
730	740	750	760	770	780
*	*	*	*	*	*
ATGGCCTCGG	TGACCGAACA	TCTGGGGCTG	GGCGCCACCA	TTTCCACCAC	CTACTACCCG
TACCGGAGCC	ACTGGCTTGT	AGACCCCGAC	CCGCGGTGGT	AAAGGTGGTG	GATGATGGGC
790	800	810	820	830	840
*	*	*	*	*	*
CCCTACCATG	TAGCCCGGGT	CGTCGCTTCG	CTGGACCAGC	TGTCCCTCCGG	GCGAGTGTCTG
GGGATGGTAC	ATCGGGCCCA	GCAGCGAAGC	GACCTGGTCTG	ACAGGAGGCC	CGCTCACAGC
850	860	870	880	890	900
*	*	*	*	*	*
TGGAACGTGG	TCACCTCGCT	CAGCAATGCA	GAGGCGCGCA	ACTTCGGCTT	CGATGAACAT
ACCTTGCAAC	AGTGGAGCGA	GTCGTTACGT	CTCCGCGCGT	TGAAGCCGAA	GCTACTTGTA
910	920	930	940	950	960
*	*	*	*	*	*
CTCGACCACG	ATGCCCCGTA	CGATCGCGCC	GATGAATTCC	TCGAGGTTCG	GCGCAAGCTC
GAGCTGGTGC	TACGGGCGAT	GCTAGCGCGG	CTACTTAAGG	AGCTCCAGCA	CGCGTTTCGAG
970	980	990	1000	1010	1020
*	*	*	*	*	*
TGGAACAGCT	GGGATCGCGA	TGCGCTGACA	CTCGACAAGG	CAACCGGCCA	GTTCCGCCGAT
ACCTTGCTCA	CCCTAGCGCT	ACGCGACTGT	GAGCTGTTCC	GTTGGCCGGT	CAAGCGGCTA
1030	1040	1050	1060	1070	1080
*	*	*	*	*	*
CCGGCTAAGG	TGCGCTACAT	CGACCACCGC	GGCGAATGGC	TCAACGTACG	CGGGCCGCTT
GGCCGATTCC	ACGCGATGTA	GCTGGTGGCG	CCGCTTACCG	AGTTGCATGC	GCCCCGCGAA
1090	1100	1110	1120	1130	1140
*	*	*	*	*	*
CAGGTGCCGC	GCTCCCCCA	GGGCGAGCCT	GTCATTCTGC	AGGCCGGGCT	TTCGGCGCGG
GTCCACGGCG	CGAGGGGGGT	CCCGCTCGGA	CAGTAAGACG	TCCGGCCCCA	AAGCCGCGCC
1150	1160	1170	1180	1190	1200
*	*	*	*	*	*
GGCAAGCGCT	TCGCCGGGCG	CTGGGCGGAC	GCGGTGTTCA	CGATTTTCGCC	CAATCTGGAC
CCGTTTCGCGA	AGCGGCCCGC	GACCCGCCCTG	CGCCACAAGT	GCTAAAGCGG	GTTAGACCTG

FIGURE 6B

1210	1220	1230	1240	1250	1260
*	*	*	*	*	*
ATCATGCAGG	CCACGTACCG	CGACATAAAG	GCGCAGGTCG	AGGCCGCGCG	ACGCGATCCC
TAGTACGTCC	GGTGCATGGC	GCTGTATTTT	CGCGTCCAGC	TCCGGCGGCC	TGCGCTAGGG
1270	1280	1290	1300	1310	1320
*	*	*	*	*	*
GAGCAGGTCA	AGGTGTTTGC	CGCGGTGATG	CCGATCCTCG	GCGAGACCGA	GGCGATCGCC
CTCGTCCAGT	TCCACAAACG	GCGCCACTAC	GGCTAGGAGC	CGCTCTGGCT	CCGCTAGCGG
1330	1340	1350	1360	1370	1380
*	*	*	*	*	*
AGGCAGCGTC	TCGAATACAT	AAATTCGCTG	GTGCATCCCC	AAGTCGGGCT	TTCTACGTTG
TCCGTCGCAG	AGCTTATGTA	TTTAAGCGAC	CACGTAGGGC	TTCAGCCCCG	AAGATGCAAC
1390	1400	1410	1420	1430	1440
*	*	*	*	*	*
TCCAGCCATG	TCGGGGTCAA	CCTTGCCGAC	TATTCGCTCG	ATACCCCGCT	GACCGAGGTC
AGGTCGGTAC	AGCCCCAGTT	GGAACGGCTG	ATAAGCGAGC	TATGGGGCGA	CTGGCTCCAG
1450	1460	1470	1480	1490	1500
*	*	*	*	*	*
CTGGGCGATC	TCGCCCAGCG	CAACGTGCCC	ACCCAAGTGG	GCATGTTTCG	CAGGATGTTG
GACCCGCTAG	AGCGGGTCGC	GTTCACCGGG	TGGGTTGACC	CGTACAAGCG	GTCTTACAAC
1510	1520	1530	1540	1550	1560
*	*	*	*	*	*
CAGGCCGAGA	CGCTGACCGT	GGGAGAAATG	GGCCGGCGTT	ATGGCGCCAA	CGTGGGCTTC
GTCCGGCTCT	GCGACTGGCA	CCCTCTTTAC	CCGGCCGCAA	TACCGCGGTT	GCACCCGAAG
1570	1580	1590	1600	1610	1620
*	*	*	*	*	*
GTCCCGCAGT	GGGCGGGAAC	CCGCGAGCAG	ATCGCGGACC	TGATCGAGAT	CCATTTCAAG
CAGGGCGTCA	CCCGCCCTTG	GGCGCTCGTC	TAGCGCCTGG	ACTAGCTCTA	GGTAAAGTTC
1630	1640	1650	1660	1670	1680
*	*	*	*	*	*
GCCGGCGGGC	CCGATGGCTT	CATCATCTCG	CCGGCGTTCC	TGCCCCGATC	TTACGAGGAA
CGGCCGCCGC	GGCTACCGAA	GTAGTAGAGC	GGCCGCAAGG	ACGGGCCTAG	AATGCTCCTT
1690	1700	1710	1720	1730	1740
*	*	*	*	*	*
TTCGTTCGATC	AGGTGGTGCC	CATCCTGCAG	CACCGCGGAC	TGTTCCGCAC	TGATTACGAA
AAGCAGCTAG	TCCACCACGG	GTAGGACGTC	GTGGCGCCTG	ACAAGGCGTG	ACTAATGCTT
1750	1760	1770	1780	1790	1800
*	*	*	*	*	*
GGCCGCACCC	TGCGCAGCCA	TCTGGGACTG	CGTGAACCCG	CATACCTGGG	AGAGTACGCA
CCGGCGTGGG	ACGCGTCGGT	AGACCCTGAC	GCACTTGGGC	GTATGGACCC	TCTCATGCGT

FIGURE 6C

Sphingomonas dsz sequence (page 4)

1810	1820	1830	1840	1850	1860
*	*	*	*	*	*
TGACGACAGA	CATCCACCCG	GCGAGCGCCG	CATCGTCGCC	GGCGGCGCGC	GCGACGATCA
ACTGCTGTCT	GTAGGTGGGC	CGCTCGCGGC	GTAGCAGCGG	CCGCCGCGCG	CGCTGCTAGT
1870	1880	1890	1900	1910	1920
*	*	*	*	*	*
CCTACAGCAA	CTGCCCCGTG	CCTAATGCCC	TGCTCGCCGC	GCTCGGCTCA	GGTATTCTGG
GGATGTCGTT	GACGGGGCAC	GGATTACGGG	ACGAGCGGCG	CGAGCCGAGT	CCATAAGACC
1930	1940	1950	1960	1970	1980
*	*	*	*	*	*
ACAGTGCCGG	GATCACACTT	GCCCTGCTGA	CCGGAAGCA	GGGCGAGGTG	CACTTCACCT
TGTCACGGCC	CTAGTGTGAA	CGGGACGACT	GGCCTTTCGT	CCCCTCCAC	GTGAAGTGGA
1990	2000	2010	2020	2030	2040
*	*	*	*	*	*
ACGACCGAGA	TGACTACACC	CGCTTCGGCG	GCGAGATTCC	GCCGCTGGTC	AGCGAGGGAC
TGCTGGCTCT	ACTGATGTGG	GCGAAGCCGC	CGCTCTAAGG	CGGCGACCAG	TCGCTCCCTG
2050	2060	2070	2080	2090	2100
*	*	*	*	*	*
TGCGTGCGCC	GGGGCGGACC	CGCCTGCTGG	GA CTGACGCC	GGTGCTGGGC	CGCTGGGGCT
ACGCACGCGG	CCCCGCTGG	GCGGACGACC	CTGACTGCGG	CCACGACCCG	GCGACCCCGA
2110	2120	2130	2140	2150	2160
*	*	*	*	*	*
ACTTCGTCCG	GGGCGACAGC	GCGATCCGCA	CCCCGGCCGA	TCTTGCCGGC	CGCCGCGTCG
TGAAGCAGGC	CCCCTGTGCG	CGCTAGGCGT	GGGGCCGGCT	AGAACGGCCG	GCGGCGCAGC
2170	2180	2190	2200	2210	2220
*	*	*	*	*	*
GAGTATCCGA	TTCGGCCAGG	AGGATATTGA	CCGGAAGGCT	GGGCGACTAC	CGCGAACTTG
CTCATAGGCT	AAGCCGGTCC	TCCTATAACT	GGCCTTCCGA	CCCCTGATG	GCGCTTGAAC
2230	2240	2250	2260	2270	2280
*	*	*	*	*	*
ATCCCTGGCG	GCAGACCCCTG	GTCGCGCTGG	GGACATGGGA	GGCGCGTGCC	TTGCTGAGCA
TAGGGACCGC	CGTCTGGGAC	CAGCGCGACC	CCTGTACCCT	CCGCGCACGG	AACGACTCGT
2290	2300	2310	2320	2330	2340
*	*	*	*	*	*
CGCTCGAGAC	GGCGGGGCTT	GGCGTCGGCG	ACGTCGAGCT	GACGCGCATC	GAGAACCCGT
GCGAGCTCTG	CCGCCCCGAA	CCGCAGCCGC	TGCAGCTCGA	CTGCGCGTAG	CTCTTGGGCA
2350	2360	2370	2380	2390	2400
*	*	*	*	*	*
TCGTCGACGT	GCCGACCGAA	CGACTGCATG	CCGCCGGCTC	GCTCAAAGGA	ACCGACCTGT
AGCAGCTGCA	CGGCTGGCTT	GCTGACGTAC	GCGGCGCGAG	CGAGTTTCCT	TGGCTGGACA

FIGURE 6D

2410 *	2420 *	2430 *	2440 *	2450 *	2460 *
TCCCCGACGT	GACCAGCCAG	CAGGCCGCAG	TCCTTGAGGA	TGAGCGCGCC	GACGCCCTGT
AGGGGCTGCA	CTGGTCGGTC	GTCCGGCGTC	AGGAAC TCCT	ACTCGCGCGG	CTGCGGGACA
2470 *	2480 *	2490 *	2500 *	2510 *	2520 *
TCGCGTGGCT	TCCCTGGGCG	GCCGAGCTCG	AGACCCGCAT	CGGTGCACGG	CCGGTCCTAG
AGCGCACCGA	AGGGACCCGC	CGGCTCGAGC	TCTGGGCGTA	GCCACGTGCC	GGCCAGGATC
2530 *	2540 *	2550 *	2560 *	2570 *	2580 *
ACCTCAGCGC	AGACGACCGC	AATGCCTATG	CGAGCACCTG	GACGGTGAGC	GCCGAGCTGG
TGGAGTCGCG	TCTGCTGGCG	TTACGGATAC	GCTCGTGGAC	CTGCCACTCG	CGGCTCGACC
2590 *	2600 *	2610 *	2620 *	2630 *	2640 *
TGGACCGGCA	CCCCGAAGTG	GTGCAGCGGC	TCGTTCGATG	CGTGGTGGAT	GCAGGGCGGT
ACCTGGCCGT	CGGGCTTGAC	CACGTCGCCG	AGCAGCTACG	GCACCACCTA	CGTCCCCGCA
2650 *	2660 *	2670 *	2680 *	2690 *	2700 *
GGGCCGAGGC	CAATGGCGAT	GTCGTCTCCC	GCCTGCACGC	CGATAACCTC	GGTGTCACTC
CCCGCTCCG	GTTACCGCTA	CAGCAGAGGG	CGGACGTGCG	GCTATTGGAG	CCACAGTCAG
2710 *	2720 *	2730 *	2740 *	2750 *	2760 *
CCGAAAGCGT	CCGCCAGGGA	TTCCGAGCCG	ATTTTCACCG	CCGCCTGACG	CCGCGGCTCG
GGCTTTCGCA	GGCGGTCCCT	AAGCCTCGGC	TAAAAGTGGC	GGCGGACTGC	GGCGCCGAGC
2770 *	2780 *	2790 *	2800 *	2810 *	2820 *
ACAGCGATGC	TATCGCCATC	CTGGAGCGTA	CTCAGCGGTT	CCTGAAGGAT	GCGAACCTGA
TGTCGCTACG	ATAGCGGTAG	GACCTCGCAT	GAGTCGCCAA	GGACTTCCTA	CGCTTGGA CT
2830 *	2840 *	2850 *	2860 *	2870 *	2880 *
TCGATCGGTC	GTTGGCGCTC	GATCGGTGGG	CTGCACCTGA	ATTCCCTCGAA	CAAAGTCTCT
AGCTAGCCAG	CAACCGCGAG	CTAGCCACCC	GACGTGGACT	TAAGGAGCTT	GTTTCAGAGA
2890 *	2900 *	2910 *	2920 *	2930 *	2940 *
CACGCCAGGT	CGAAGGGCAG	ATAGCATGAA	CGAACTCGTC	AAAGATCTCG	GCCTCAATCG
GTGCGGTCCA	GCTTCCCGTC	TATCGTACTT	GCTTGAGCAG	TTTCTAGAGC	CGGAGTTAGC
2950 *	2960 *	2970 *	2980 *	2990 *	3000 *
ATCCGATCCG	ATCGGCGCTG	TGCGGCGACT	GGCCGCGCAG	TGGGGGGCCA	CCGCTGTTGA
TAGGCTAGGC	TAGCCGCGAC	ACGCCGCTGA	CCGGCGCGTC	ACCCCCCGGT	GGCGACAACT

FIGURE 6E

3010	3020	3030	3040	3050	3060
*	*	*	*	*	*
TCGGGACCGG	GCCGGCGGAT	CGGCAACCGC	CGAACTCGAT	CAACTGCGCG	GCAGCGGCCT
AGCCCTGGCC	CGGCCGCGCTA	GCCGTTGGCG	GCTTGAGCTA	GTTGACGCGC	CGTCGCCGGA
3070	3080	3090	3100	3110	3120
*	*	*	*	*	*
GCTCTCGCTG	TCCATTCCCG	CCGCATATGG	CGGCTGGGGC	GCCGACTGGC	CAACGACTCT
CGAGAGCGAC	AGGTAAGGGC	GGCGTATACC	GCCGACCCCG	CGGCTGACCG	GTTGCTGAGA
3130	3140	3150	3160	3170	3180
*	*	*	*	*	*
GGAAGTTATC	CGCGAAGTCG	CAACGGTGGG	CGGATCGCTG	GCGCATCTAT	TCGGCTACCA
CCTTCAATAG	GCGCTTCAGC	GTTGCCACCT	GCCTAGCGAC	CGCGTAGATA	AGCCGATGGT
3190	3200	3210	3220	3230	3240
*	*	*	*	*	*
CCTCGGCTGC	GTACCGATGA	TCGAGCTGTT	CGGCTCGGCG	CCACAAAAGG	AACGGCTGTA
GGAGCCGACG	CATGGCTACT	AGCTCGACAA	GCCGAGCCGC	GGTGTTTTCC	TTGCCGACAT
3250	3260	3270	3280	3290	3300
*	*	*	*	*	*
CCGCCAGATC	GCAAGCCATG	ATTGGCGGGT	CGGGAATGCG	TCGAGCGAAA	ACAACAGCCA
GGCGGTCTAG	CGTTCGGTAC	TAACCGCCCA	GCCCTTACGC	AGCTCGCTTT	TGTTGTCGGT
3310	3320	3330	3340	3350	3360
*	*	*	*	*	*
CGTGCTCGAG	TGGAAGCTTG	CCGCCACCGC	CGTCGATGAT	GGCGGGTTTC	TCCTCAACGG
GCACGAGCTC	ACCTTCGAAC	GGCGGTGGCG	GCAGCTACTA	CCGCCCAAGC	AGGAGTTGCC
3370	3380	3390	3400	3410	3420
*	*	*	*	*	*
CGCGAAGCAC	TTCTGCAGCG	GCGCCAAAAG	CTCCGACCTG	CTCATCGTGT	TCGGCGTGAT
GCGCTTCGTG	AAGACGTCGC	CGCGGTTTTC	GAGGCTGGAC	GAGTAGCACA	AGCCGCACTA
3430	3440	3450	3460	3470	3480
*	*	*	*	*	*
CCAGGACGAA	TCCCCCTGC	GCGGCGCGAT	CATCACCGCG	GTCATTCCCA	CCGACCGGGC
GGTCCTGCTT	AGGGGGGACG	CGCCGCGCTA	GTAGTGCGCG	CAGTAAGGGT	GGCTGGCCCG
3490	3500	3510	3520	3530	3540
*	*	*	*	*	*
CGGTGTTTCAG	ATCAATGACG	ACTGGCGCGC	AATCGGGATG	CGCCAGACCG	ACAGCGGCAG
GCCACAAGTC	TAGTTACTGC	TGACCGCGCG	TTAGCCCTAC	GCGGTCTGGC	TGTCGCCGTC
3550	3560	3570	3580	3590	3600
*	*	*	*	*	*
CGCCGAATTT	CGCGACGTCC	GAGTCTACCC	AGACGAGATC	TTGGGGGCAC	CAAACCTCAGT
GCGGCTTAAA	GCGCTGCAGG	CTCAGATGGG	TCTGCTCTAG	AACCCCGTG	GTTTGAGTCA

FIGURE 6F

3610	3620	3630	3640	3650	3660
*	*	*	*	*	*
CGTTGAGGCG	TTCGTGACAA	GCAACCGCGG	CAGCCTGTGG	ACGCCGGCGA	TTCAGTCGAT
GCAACTCCGC	AAGCACTGTT	CGTTGGCGCC	GTCGGACACC	TGCGGCCGCT	AAGTCAGCTA
3670	3680	3690	3700	3710	3720
*	*	*	*	*	*
CTTCTCGAAC	GTTTATCTGG	GGCTCGCGCG	TGGCGCGCTC	GAGGCGGCAG	CGGATTACAC
GAAGAGCTTG	CAAATAGACC	CCGAGCGCGC	ACCGCGCGAG	CTCCGCCGTC	GCCTAATGTG
3730	3740	3750	3760	3770	3780
*	*	*	*	*	*
CCGGACCCAG	AGCCGCCCCCT	GGACACCCGC	CGGCGTGGCG	AAGGCGACAG	AGGATCCCCA
GGCCTGGGTC	TCGGCGGGGA	CCTGTGGGCG	GCCGCACCGC	TTCCGCTGTC	TCCTAGGGGT
3790	3800	3810	3820	3830	3840
*	*	*	*	*	*
CATCATCGCC	ACCTACGGTG	AACTGGCGAT	CGCGCTCCAG	GGCGCCGAGG	CGGCCGCGCG
GTAGTAGCGG	TGGATGCCAC	TTGACCGCTA	GCGCGAGGTC	CCGCGGCTCC	GCCGCGCGCG
3850	3860	3870	3880	3890	3900
*	*	*	*	*	*
CGAGGTCGCG	GCCCTGTTGC	AACAGGCGTG	GGACAAGGGC	GATGCGGTGA	CGCCCCAAGA
GCTCCAGCGC	CGGGACAACG	TTGTCCGCAC	CCTGTTCCCG	CTACGCCACT	GCGGGCTTCT
3910	3920	3930	3940	3950	3960
*	*	*	*	*	*
GCGCGGCCAG	CTGATGGTGA	AGGTTTCGGG	TGTGAAGGCC	CTCTCGACGA	AGGCCGCCCT
CGCGCCGGTC	GACTACCACT	TCCAAAGCCC	ACACTTCCGG	GAGAGCTGCT	TCCGGCGGGA
3970	3980	3990	4000	4010	4020
*	*	*	*	*	*
CGACATCACC	AGCCGTATTT	TCGAGACAAC	GGGCTCGCGA	TCGACGCATC	CCAGATACGG
GCTGTAGTGG	TCGGCATAAA	AGCTCTGTTG	CCCAGCGCT	AGCTGCGTAG	GGTCTATGCC
4030	4040	4050	4060	4070	4080
*	*	*	*	*	*
ATTCGATCGG	TTCTGGCGTA	ACATCCGGAC	TCATACGCTG	CACGATCCGG	TATCGTATAA
TAAGCTAGCC	AAGACCGCAT	TGTAGGCCTG	AGTATGCGAC	GTGCTAGGCC	ATAGCATATT
4090	4100	4110	4120	4130	4140
*	*	*	*	*	*
AATCGTCGAT	GTGGGGAAC	ACACGCTCAA	CGGGACATTC	CCGGTTCCCG	GATTTACGTC
TTAGCAGCTA	CACCCCTTGA	TGTGCGAGTT	GCCCTGTAAG	GGCCAAGGGC	CTAAATGCAG

ATGA
TACT

FIGURE 6G

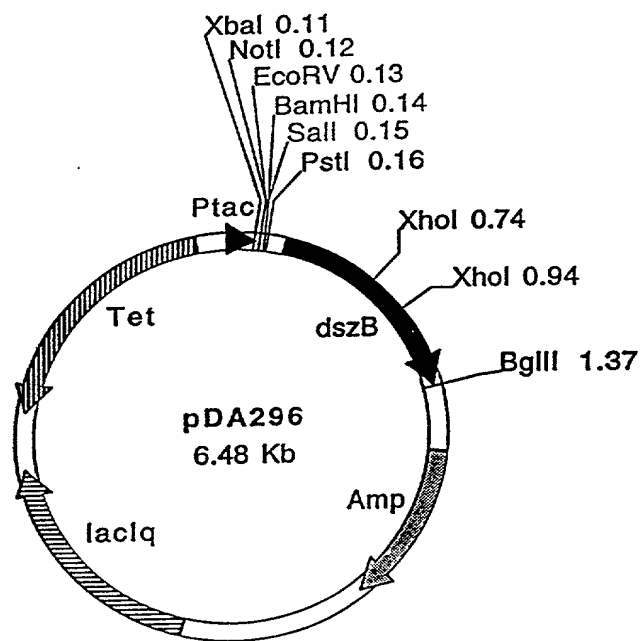


FIGURE 7

DszA (S) 1 MTDPRQLHLAGFFCAGNVTHAHGAWRHADDSNGFLTKEYYQQIARTLERG 50
 ||: . ||: |||||: ||||| ||||| . ||: ||. . ||: |||||

DszA (R) 1 MTQQRQMHLAGFFSAGNVTHAHGAWRHTDASNDFLSGKYYQHIARTLERG 50

DszA (S) 51 KFDLLFLPDALAVWDSYGDNLETGLRYGGQGAVMLEPGVVIAAMASVTEH 100
 ||||| |||||: ||| |||||: ||: . ||||| |||: ||: ||. |||||

DszA (R) 51 KFDLLFLPDGLAVEDSYGDNLDTGVGLGGQGAVALEPASVVATMAAVTEH 100

DszA (S) 101 LGLGATISTTYPPYHVARVVASLDQLSSGRVSWNVVTSLSNAEARNFGF 150
 ||||| ||||| . ||||| ||||| . |||||: ||||| ||||| . |||||: |||||

DszA (R) 101 LGLGATISATYPPYHVARVFATLDQLSGGRVSWNVVTSLNDAEARNFGI 150

DszA (S) 151 DEHLHDARYDRADEFLEVVRKLWNSWDRDALTLDKATGQFADPAKVRYI 200
 : ||: ||||| ||||| . ||: ||||| ||| . |||| . || |||||: ||:

DszA (R) 151 NQHLEHDARYDRADEFLEAVKKLWNSWDEDALVLDKAAGVFADPAKVHYV 200

DszA (S) 201 DHRGEWLNVRGPLQVPRSPQGEFVILQAGLSARGKRFAGRWADAVFTISP 250
 ||: ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||

DszA (R) 201 DHHGEWLNVRGPLQVPRSPQGEFVILQAGLSRPRGRRFAGKWAEAVFSLAP 250

DszA (S) 251 NLDIMQATYRDIKAQVEAAGRDPEQVKVFAAVMPILGETEAIARQREYI 300
 ||: ||||| . ||: ||: |||||: ||. ||: |||||: ||||| . ||: ||: |||||:

DszA (R) 251 NLEVMOATYQGIKAEVDAAGRDPDQTKIFTAVMPVLGESQAVAQERLEYL 300

DszA (S) 301 NSLVHPEVGLSTLSSHVGVNLDYSLDTPLTEVLGDLAQRNVPTQLGMFA 350
 ||||| ||||| ||||| . ||: |||| . |||||: ||: ||||| ||||| ||||| |||||

DszA (R) 301 NSLVHPEVGLSTLSSHTGINLAAYPLDTPIKDILRDLQDRNVPTQLHMFA 350

DszA (S) 351 RMLQAETLTVGEMGRRYGANVGFPQWAGTREQIADLIEIHFKAGGADGF 400
 : . ||: ||: ||||| . ||||| ||||| ||||| : ||: ||: |||||

DszA (R) 351 AATHSEELTLAEMGRRYGTNVGFVPQWAGTGEQIADELIRHFEGGAADGF 400

DszA (S) 401 IISPAFLPGSYEEFVDQVVPILQHRGLFRDYEGRTLRLSHLGLREPAYLG 450
 ||||| |||||: ||||| |||||: ||. ||. ||||: ||: |||| . ||||| ||.. ||

DszA (R) 401 IISPAFLPGSYDEFVDQVVPVLQDRGYFRTEYQGNLRLDHLGLRVPQLQG 450

DszA (S) 451 EYA 453
 : .

DszA (R) 451 QPS 453

FIGURE 8

DszB(S)	MTTDIHPASAASSPAA--RATITYSNCPVPNALLAALGSGILDSAGITLALL	50
DszB	MTSRVDPANPGSELDSAIRDTLTYSNCPVPNALLTASESGFLDAAGIELDVL	52
DszB(S)	TGKQGEVHFTYDRDDYTRFGGEIPPLVSEGLRAPGRTRLLGLTPVLGRWGYF	102
DszB	SGQQGTVHFTYDQPAYTRFGGEIPPLSEGLRAPGRTRLLGITPLLGRQGFF	104
DszB(S)	VRGDSAIRTPADLAGRRVGVSDSARRILTGRIGDYRELDPWQTLVALGTWE	154
DszB	VRDDSPITAAADLAGRRIGVSASAIRILRGQLGDYLELDPWQTLVALGSWE	156
DszB(S)	ARALLSTLETAGLGVGDVELTRIENTPFVDVPTERLHAAGSLKGTDLFPDVTS	206
DszB	ARALLHTLEHGELGVDDVELVPISSPGVDVPAEQLEESATVKGADLFPDVAR	208
DszB(S)	QOAAVLEDERADALFAWL PWAAELETRIGARPVLDLSADDRNAYASTWTVSA	258
DszB	GQAAVLASGDVDALYSWLPWAGELQA-TGARPVVDLGLDERNAYASVWTVSS	260
DszB(S)	ELVDRQPELVQRLVDAVVDAGRWAEEANGDVVSRLHADNLGVSPESVRQGFGA	310
DszB	GLVRQRPGLVQRLVDAAVDAGLWARDHSDAVTSLHAANLGVSTGAVGQGFGA	312
DszB(S)	DFHRLTPRLDSDAIAILERTQRFKLDANLIDRSLALDRWAAPEFLEQSLSRQVEGQIA	369
DszB	DFQQRLVPRLDHDAIALLERTQQFLLTNLLQEPVALDQWAAPEFLNNSLNRHR	365

FIGURE 9

DszC(S) 1MNELVKDLGLNRS DPIGAVRR LAAQWGATAVDRDRAGGSATAELD 45
 DszC(R) 1 MTLSPKQHVPRDAADNDPVAVARGLAEKWRATAVERDRAGGSATAERE 50
 DszC(S) 46 QLRGSGLLSL SIPAAYGGWGADWPTTLEVIREVATVDGSLAHLFGYHLGC 95
 DszC(R) 51 DLRASALLSL LVPREYGGWGADWPTAIEVVREIAAADGSLGHLFGYHLTN 100
 DszC(S) 96 VPMIELFGSAPQKERLYRQIASHDWRVGNASSENNSHVLEWKLAATAVDD 145
 DszC(R) 101 APMIELIGSQEQEEHLYTQIAQNNWWTGNASSENNSHVLWDKVSATPTED 150
 DszC(S) 146 GGFVLNGAKHFCSGAKSSDLLIVFGVIQDESPLRGAIITAVIPTDRAGVQ 195
 DszC(R) 151 GGYVLNGTKHFCSGAKGSDLLFVFGVVQDDSPQQGAIIAAAIPTSRAGVT 200
 DszC(S) 196 INDDWRAIGMRQTDSGSAEFRDVRVYPDEILGAPNSVVEAFVTSNRGSLW 245
 DszC(R) 201 PNDDWAAIGMRQTDSGSTDFHNVKVEPDEV LGAPNAFVLAFIQSERGSLF 250
 DszC(S) 246 TPAIQSIFS NVYLGLARGALEAAADYTRTQSRPWTPAGVAKATEDPHIIA 295
 DszC(R) 251 APIAQLIFANVYLGLIAHGALDAAREYTRTQARPWTPAGIQQATEDPYTIR 300
 DszC(S) 296 TYGELAIALQGAEEAAAREVAALLOQAWDKGDAVTPEERGQLMVKVS GVKA 345
 DszC(R) 301 SYGEFTIALQGADAAAREAAHLLQTVWDKGDALTPEDRGELMVKVS GVKA 350
 DszC(S) 346 LSTKAALDITSRIFETTGSRSTHPRYGFDRFWRNIRTHTLHDPVSYKIVD 395
 DszC(R) 351 LATNAALNISSGVFEVIGARGTHPRYGFDRFWRNVRTSLHDPVSYKIAD 400
 DszC(S) 396 VGNYTLNGTFPVP GF TS 412
 DszC(R) 401 VGKHTLNGQYPIPGF TS 417

FIGURE 10

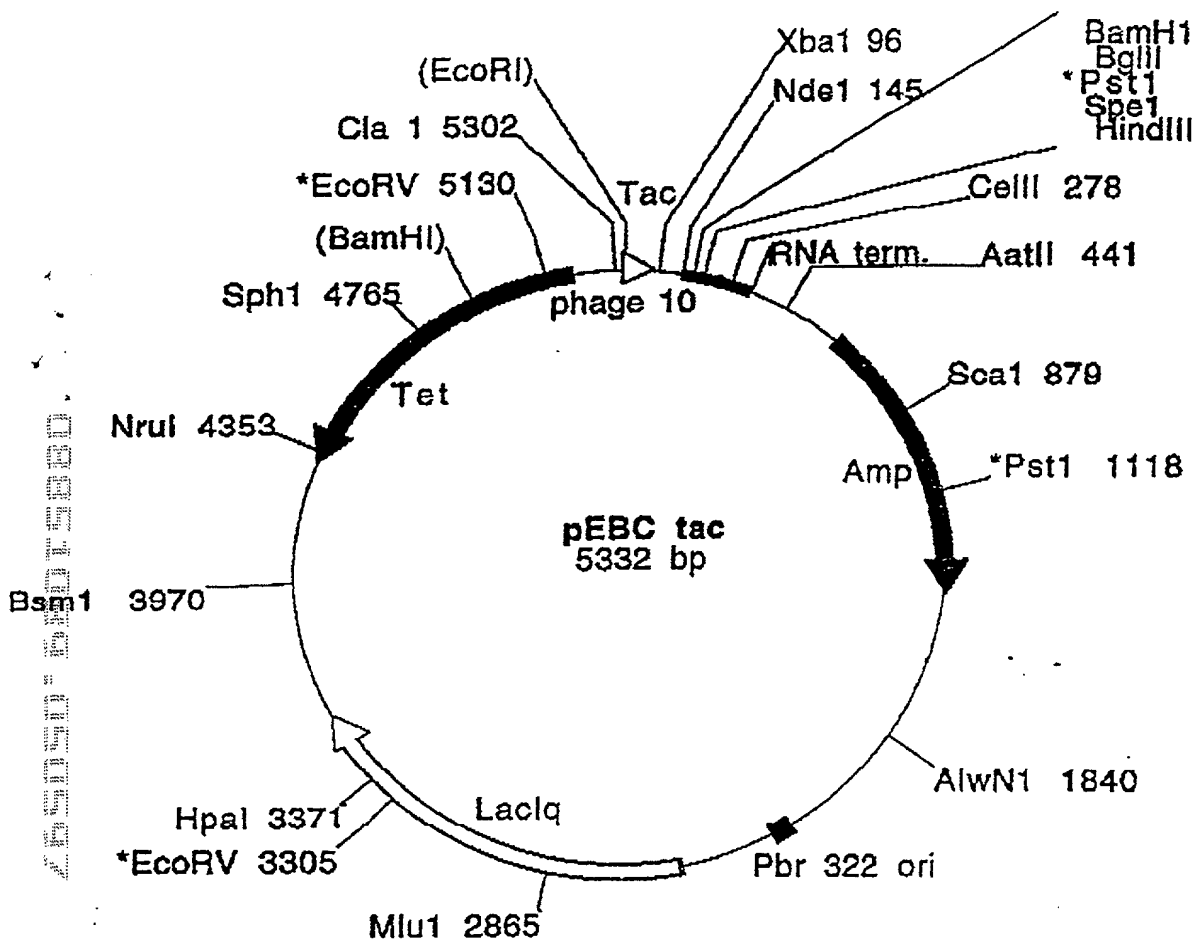


FIGURE 11

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

A SPHINGOMONAS BIODESULFURIZATION CATALYST

the specification of which (check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application

Number or PCT International Application Serial No. _____

and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

			Priority Not Claim
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

_____ (Application Number)	_____ (Filing Date)
_____ (Application Number)	_____ (Filing Date)

Attorney

(Application Serial No.)	(Filing date)	(Status, patented, pending, abandoned)

I also hereby grant additional Powers of Attorney to the following attorney(s) and/or agent(s) to file and prosecute an international application under the Patent Cooperation Treaty based upon the above-identified application, including a power to meet all designated office requirements for designated states:

David E. Brook	Reg. No. 22,592	Thomas O. Hoover	Reg. No. 32,470
James M. Smith	Reg. No. 28,043	Alice O. Carroll	Reg. No. 33,542
Leo R. Reynolds	Reg. No. 20,884	N. Scott Pierce	Reg. No. 34,900
Patricia Granahan	Reg. No. 32,227	Richard A. Wise	Reg. No. 18,041
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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